THE USE OF EXPERIMENTAL INFECTION MODELS TO INVESTIGATE THE CORRELATION BETWEEN CLINICAL AND PATHOLOGICAL MEASURES OF THE SEVERITY OF RESPIRATORY DISEASE IN THREE SPECIES

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The use of experimental infection models to investigate the correlation between clinical and pathological measures of the severity of respiratory disease in three species

Abstract

The objective of this research was to investigate the relationship between *ante mortem* assessments of disease severity and the gross pathological manifestations of disease observed during necropsy. Many clinical assessments are subjective, the hypothesis under investigation is that these correlate with the pathological state of the animal.

Bronchopneumonia due to *Pasteurella haemolytica* A1 in calves, *Actinobacillus pleuropneumoniae* infection in pigs, and *Mycoplasma gallisepticum* infection of chickens were investigated. The diseases were induced by experimental infection and animals were closely monitored. For calves and pigs, rectal temperature, respiratory rates and clinical demeanour scores were recorded and at necropsy, the respiratory tract and any other affected organs examined. For chickens, an auscultated respiratory score was used, serological responses were recorded and, upon necropsy, inspection of airsac lesions and mycoplasma isolation were performed.

Both graphical and statistical methods were used to correlate *ante mortem* measures of disease severity with each other and with *post mortem* measures of gross pathological lesions and bacteriological isolation of the infecting organism. Calves showed highly correlated relationship between *ante mortem* and *post mortem* indicators of disease severity. For pigs, the relationship between clinical variables and gross pathology was much less clear and varied between the three serotypes of *Actinobacillus pleuropneumoniae* used (serotypes 3, 5a, 9). In chickens, there were few clinical signs evident until the pathology compromised the physiological reserve capacity of the airsacs. Beyond this 'threshold', rapid increases in respiratory auscultation score, mycoplasma isolation and serological response were all indicators of increasing gross pathological changes.

The relationships between *ante mortem* assessments of disease severity and the gross pathological lesions recorded at necropsy were usually non-linear and differed for each pair of variables correlated. The strength of correlation between pairs of variables also differed according to the stage of disease progression and severity of the infective episode for each of the species studied. The relationships between clinical variables being used as indicators of disease severity and the *post mortem* pathological measures need to be clearly understood for each variable before conclusions concerning the disease severity, prognosis or, in treated animals, efficacy predictions can be made.

Keywords: clinical score, disease severity, efficacy assessment, experimental infection, respiratory disease, *Pasteurella haemolytica, Actinobacillus pleuropneumoniae, Mycoplasma gallisepticum*

Statement of originality

The compilation of this thesis and the work contained therein has been conducted by the author and has not been submitted in candidature of another qualification. Where relevant, acknowledgement has been made of collaboration with colleagues. The clinical work and data collection have been conducted in accordance with the European Union Guidelines for Good Clinical Practice for the conduct of clinical trials for Veterinary Medicinal Products (GCPV) (Anon 1994).

Lloyd. G. Reeve-Johnson
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Lloyd. G. Reeve-Johnson

CHAPTER 1: INTRODUCTION TO THE THESIS

Introduction

The intention of this research is to investigate the assessment of disease severity for respiratory infections using experimental infection studies. In the assessment of disease severity, scoring systems are routinely used to quantitatively express the clinical condition and the clinical progression of animals. There is little literature discussing the correlation of clinical scoring systems with pathological measures of disease severity. In this research, respiratory diseases have been induced by experimental infection. The clinical signs of animals have been recorded and correlated with pathological observations upon necropsy. Experimental infections were conducted in calves, chickens and pigs, which are the three most economically important farm animal species in European agriculture.

The assessment of overall health status of any living animal is very subjective. Individual clinicians ascribe different levels of importance to each disease sign (Espinasse *et al.* 1989). A clinical evaluation depends on a variety of factors including the severity, type and duration of different signs, as well as patient-specific factors such as age, immune and nutritional status. In human medicine clinical scoring has been used as an aid to giving a prognosis e.g. patients infected with human immuno-deficiency virus (Vanhems and Toma 1995) as well as an indicative diagnostic measure (Park *et al.* 1991, Herrera and Jepson 1994). In veterinary medicine use, according to the literature, is largely restricted to a measure of the severity of the disease at a point in time (Ishibashi and Matsumoto 1985, Robb and Kronfield 1986), or the clinical response over a period of time (O'Dair *et al.* 1994, Back *et al.* 1995). The weighting and importance ascribed to each disease sign used in constructing a clinical scoring system generally depends upon the experience of the clinician (Espinasse *et al.* 1989). The overall condition of the animal is also an important determinant of the impact that any pathological process will have (Thrusfield 1995, Reeve-Johnson 1997).

The assessment of clinical condition is largely subjective and qualitative. Scoring systems provide a method of transposing clinical assessments into more objective and quantitative records in order that comparisons between patients, and improvements in individual patients can be communicated in scientific literature and between clinicians.

A clinical score is usually a numeric representation of the severity of clinical signs. The capacity of a scoring system to grade severity of signs depends upon the scale, which in the crudest form is absent or present (0 or 1) e.g. absence or presence of a cough or naso-ocular discharge, but may also further attempt to grade severity of signs by grouping them into categories of severity e.g. mild, moderate and severe. The parameters taken into account when producing a clinical score vary according to the body system which is being examined and the number of easily recognisable signs which are consistently present and are indicative of the disease under observation. Usually there is an underlying assumption in clinical scoring systems that the appearance of signs coincide with the pathological progression of disease and that the more signs present, the more severe the disease. However, the appearance of overt clinical signs does not give a linear and evenly graduated representation of the underlying pathological progression. There is little veterinary literature which attempts to correlate clinical findings with observed pathology, and no statistically valid controlled studies appear to have been conducted. Part of the reason is that, in the field, animals are generally monitored until recovery and are not submitted for necropsy unless severely diseased. With field cases, which usually are of multi-factorial aetiology, unless animals come to necropsy during the acute disease episode, pathological changes cannot always be readily ascribed to a particular pathogen or even pathological episode.

In order to better understand how clinical signs reflect the pathological progression of respiratory disease, statistical evaluation of the correlation between clinical and pathological signs was conducted in three species suffering from distinct respiratory conditions, each of significant economic impact to the animal production industry.

The conditions investigated were: respiratory pasteurellosis in calves ('shipping fever') as caused by *Pasteurella haemolytica* both in combination with *Mycoplasma bovis*, and as the sole pathogen; in chickens, mycoplasmosis as caused by *Mycoplasma gallisepticum*; and in pigs, Actinobacillosis caused by serotypes 3, 5a and 9 of *Actinobacillus pleuropneumoniae.*

The intent of this work is to develop a better understanding of how clinical scoring systems relate to the gross pathology observed at necropsy. Additionally, the correlation between these clinical scoring systems and other variables, such as cough score in pigs (MacKinnon 1998) or rectal temperature in calves (Scott 1996, 1998) which have been suggested as being more reliable objective measures of the onset of clinical disease in animals was investigated.

Background

Qualitative clinical scoring systems have long been used by the medical profession as a means of communicating the severity of a patient's condition, for a variety of diseases e.g. cancer (Karnofsky and Burchenal 1949), lower respiratory tract infections (Silverman and Anderson 1956, Kjolhede *et al.* 1995), bowel conditions such as Crohn's disease (Park *et al.* 1991), eczema (Glover and Atherton 1992), malaria (Herrera and Jepson 1994) and HIV (Vanhems and Toma 1995). Quantitative indicators of clinical status include temperature, respiratory rate and blood pressure. Different scoring systems assess different factors, some concentrating upon single signs (Back *et al.* 1995) and others being based upon a combination of signs (Magnaval 1995).

In veterinary medicine, scoring systems are similarly applied but of necessity are adapted according to the species. Consequently, many different systems have been used to describe clinical demeanour in animals. The background literature review has included examples from pigs (Fedorka-Cray *et al.* 1993), calves (Gourlay *et al.* 1989, Thomas *et al.* 1989, Braidwood and Henry 1990, Genicot *et al.* 1993), adult bovines (Collie 1992), horses (Robb and Kronfield 1986, Milne *et al.* 1994), dogs (Sture and Lloyd 1995), cats (O'Dair *et al.* 1994), and rabbits (Ishibashi and Matsumoto 1985).

No literature making direct correlations between the *ante mortem* clinical score and gross organ pathology appears to exist. Some authors have commented on the 'significant heterogenous variance' in clinical scores (Moore *et al.* 1996) indicating the influence that environment, infection pressure, immune status of different groups of animals, or even genetic makeup, might have on the clinical condition as assessed by a scoring system, but this has not been investigated in relation to pathological findings at necropsy. In order for the pathogenesis of disease to be more fully understood, clinical trial conditions which can be closely controlled and monitored are needed and the ability to gather *ante* and *post mortem* data must exist. Experimental infection studies which were being developed for the future efficacy testing of vaccines and antimicrobials presented such an opportunity.

Research Objectives

Experimental infection studies involving respiratory disease in calves, chickens and pigs were conducted. In order to control the type and severity of respiratory disease, animals were inoculated with a pre-defined dose of infecting organism by an established route of inoculation. The clinical scores and pathological measurements were standardised for all trials involving a single species.

A literature review was conducted to locate disease models which had been extensively used and which produced an acceptable level of repeatability, in each of the study species. Based upon this review, pilot studies and model development work were conducted to ensure that the experimental infection models worked reliably and to modify the methodology where appropriate. The author conducted all the literature searches, designed the study protocols, supervised and was closely involved with the clinical conduct of each study, collated and interpreted the data. Statistical advice was sought from specialist statisticians. Staff based permanently on the trial sites were responsible for handling non-planned incidents, and these were promptly communicated to the author.

The objectives of this research were:

1. To reproduce respiratory disease under defined conditions, as caused by distinct and economically important pathogens in calves, chickens and pigs.
2. To monitor the clinical course of these diseases and to quantify the clinical signs of subjects by means of pre-determined clinical scoring systems.
3. To conduct a detailed *post mortem* inspection of the respiratory tract and any other affected organs of animals upon necropsy.
4. To analyse the correlation between the clinical signs exhibited by animals prior to necropsy and the gross pathological signs during necropsy shortly thereafter.

Chapter 2: Experimental infection of calves and the evaluation of disease severity

Introduction

Infectious pneumonia caused by viruses, mycoplasmas and bacteria constitute a major problem in countries all over the world. Estimates of economic loss attributable to infectious respiratory disease in young cattle have been performed (Thomas 1977, Loan 1984, Gourlay *et al.* 1989, Jones 1992, Virtala *et al.* 1996a, Reeve-Johnson *et al.* 1997b, Esslemont *et al.* 1998, Reeve-Johnson 1998b). In each case, there were very large economic losses in production. Thomas *et al.* (1978) using data from the Milk Marketing Board's beef progeny testing scheme, concluded that enzootic pneumonia, pneumonic consolidation and pleurisy together account for the majority of losses from fatal disease. Scott (1998) has stated that respiratory disease is the major cause of financial loss due to infectious disease in intensively reared cattle units. In 1989, enzootic pneumonia in weaned calves was estimated to cost British farmers in excess of £50 million annually (Gourlay *et al.* 1989). Jones (1992) estimated that the viral component of respiratory disease was responsible for £60 million annually in the UK. Virtala *et al.* (1996a) using linear regression models demonstrated that pneumonia can reduce body-weight gain of female dairy calves by 800g per week during the first three months of life which has an additional impact on the age of first calving. Reeve-Johnson (1998b) reported that as many as 16 million cattle may be affected annually with respiratory disease in Europe and that non-fatal production losses probably cost the European cattle industry as much as ECU 576 million annually. Esslemont *et al.* (1998) performed a range of illustrations on the costs of respiratory diseases in dairy heifers. With a typical case of 30% morbidity and 5% mortality, the total costs were found to amount to £3824-£4732 per 100 cases (£830 for veterinary treatments, £300 for veterinarian's time, £2240 for depression of the calf's performance and up to £1362 for the loss of stockperson's time).

The aetiology of calf bronchopneumonia is complex and can involve viruses, mycoplasmas and bacteria (Dyer 1982, Wikse 1990) as well as managemental and environmental factors (Frank 1986, Cole 1996, Grandin 1996, Perino 1996). Primary viral infections may independently cause clinical disease, depending on the virus and the condition of the host. More importantly, they predispose the animal to secondary complications such as bacterial invasion, particularly when other immunosuppressive factors are present. Such factors include poor nutrition, stress (e.g due to transportation, handling, de-horning and castration) and concurrent disease. The immunosuppressive effects of some bovine viral pathogens are manifested in their ability to facilitate bacterial colonisation of the lower respiratory tract (Jakab 1984). This has been demonstrated with several viruses including bovine herpes virus 1 (Jericho and Langford 1978), bovine viral diarrhoea virus (Corstvet and Panciera 1982), para-influenza-3 virus (Baldwin *et al.* 1967) and bovine respiratory syncytial virus (Lehmkuhl and Gough 1977). There is no information about the specific immuno-suppressive effects for many of the other viruses isolated from bovine lungs such as bovine adenovirus, bovine enterovirus, bovine parvovirus, caronavirus, calicivirus, bovine reovirus, bovine rhinovirus, malignant catarrhal fever virus and bovine herpes virus 4 (Perino 1996). At present in the UK, the viruses most commonly demonstrated in outbreaks of pneumonia are para-influenza 3, respiratory syncytial virus, calf coronavirus, bovine herpes virus 1 and bovine viral diarrhoea/mucosal disease virus.

Bacteria, particularly *Pasteurella spp.*, play an important role in many outbreaks of calf bronchopneumonia. They increase the severity of the primary lung damage caused by viruses and exacerbate the clinical signs, frequently with a fatal outcome, due to leukotoxin release. Furthermore, experimental studies indicate that *Pasteurella haemolytica* can act as a primary pathogen, producing severe acute pneumonia in calves (Confer *et al.* 1984, Gibbs *et al.* 1984, Ames *et al.* 1985).

Chlamydia spp. also play a role as bovine respiratory pathogens. Clinical disease resulting from experimental infection with *P.haemolytica* has been shown to be more severe when *Chlamydia* is added to the challenge (Palotay and Christensen 1959). *Haemophilus somnus* is an important cause of respiratory disease in feedlot cattle in North America. Although pnemonia due to this organism is increasingly being reported in Europe, enzootic calf pneumonia and pneumonic pasteurellosis remain the most important causes of respiratory disease in Europe (Bryson 1996).

Mycoplasmas have also been demonstrated to play a role in the development of respiratory disease. Evidence suggests that they compromise the innate defences of the host including the mucociliary escalator, bovine alveoar macrophages and neutrophil function (Jarstrand *et al.* 1975, Thomas *et al.* 1991, Almeida *et al.* 1992, Reeve-Johnson *et al.* 1997b, Reeve-Johnson 1998b). A study on the epidemiological and pathological characteristics of respiratory tract disease in dairy heifers during the first three months of life suggested a synergistic effect between *Mycoplasma spp.* and *P.multocida*. *Mycoplasma spp.* were isolated from 75% of animals showing clinical signs of respiratory disease and *Pasteurella spp.* were isolated from 69 percent (Virtala *et al.* 1996b).

Three experimental infection studies were used to reproduce respiratory pasteurellosis in calves due to either *Pasteurella haemolytica* Type A1 on its own, or in combination with concurrent *Mycoplasma bovis* infection. The variables measured as indicators of disease severity were: clinical demeanour score, respiratory rate, rectal temperature, percent lung consolidation and microbiological reisolation.

Materials and methods

Calves aged eight to thirteen days, which had been reared in closed herds, were introduced to the experimental animal houses. Room temperatures were approximately 20°C and humidity and ventilation were similar for each study. Identification of calves was by means of uniquely numbered ear tags.

At intake, animals were examined for general health and nasopharyngeal swabs were examined by bacterial culture for presence of adventitious infection with *P.haemolytica* and *M.bovis*. Calves were observed closely during the rearing period to ensure adequate milk intake and for evidence of incidental diseases.

Calves were weighed and allocated to individual pens with no direct contact between animals. After introducing calves to the experimental animal house, they were fed a glucose/electrolyte solution for two feeds before being reared on a reconstituted commercial milk replacer powder. Calves were bucket-fed each morning and afternoon at the same time and were not fed any additional concentrated feed. Milk refusals were scored.

Once calves were acclimatised, they were experimentally infected according to the schedule described for each study. Clinical demeanour scores, rectal temperature, respiratory rate, mortality, percent lung consolidation and microbiological reisolation were recorded. The studies ended seven days after infection and necropsy was conducted on all calves.

Measurement of disease severity

Measurement of rectal temperature has been cited as an objective measure of clinical condition in calves. Different authors consider different thresholds for rectal temperature to be indicative of bovine respiratory disease. These range from 39.0°C (Laurisden *et al.* 1994) to >40.5°C (Mechor *et al.* 1988, Gorham *et al.* 1990, Picavet *et al.* 1991, Morck *et al.* 1993). Scott (1994, 1996, 1998) however argues that 39.5°C is a valid threshold for detecting early onset of respiratory disease as the cost effectiveness of treatment is greater if treatment is initiated once rectal temperatures exceed 39.5°C. In this set of studies, rectal temperature was monitored, in conjunction with respiratory rate and clinical demeanour score, in an attempt to understand the relationship between these variables used as indicators of disease severity.

Rectal temperature

Eight clinical thermometers were placed in a water bath at 25°C, 37°C and 45 °C, there was little variation between the thermometers, but three which recorded identical readings were selected for use in the studies. Rectal temperatures were recorded at approximately the same time, in each study, 30 minutes before feeding.

Clinical demeanour score

Through repeated observation of calves, a scoring system was developed which could be practically applied to farm situations to grade the overall clinical demeanour of calves. A high level of variation was noted between experienced clinicians in interpreting disease signs in calves as mild, moderate or severe, and confusion between the grading of individual signs as opposed to the overall demeanour of the animal was frequent. In an attempt to reduce this, the terms 'normal', 'subdued', 'apathy' or 'severe disease' were used and further descriptive details were added to help classify calves and to attempt to increase the uniformity of application of this proposed scoring method (Reeve-Johnson and Otte, 1998). While the majority of observations were recorded by the author, for each study an additional observer was also trained in using the system. Once familiar with the system, there was a high level of consistency when observations were compared with those conducted independently but at the same time by the author.

0	"Normal"	Alert calf, responds to presence of observer, head turns, ears pricked, bright eyes and lustrous coat. No signs of disease evident.
1	"Subdued"	Less bright and alert than normal calf, may cough intermittently or show other signs of disease upon careful observation, such as nasal or ocular discharges and appetite may be reduced.
2	"Apathy"	Responds slowly to presence of observer, ears turned down, coat losing lustre, disease signs immediately apparent, such as coughing, nasal or ocular discharges, appetite reduced.
3	"Severe disease"	Fails to respond to observer, inappetant.

Respiratory rate

In the first study, this was recorded as a score:

	Breaths per minute
1	40-50
2	51-59
3	60-80

For interpretation, the intermediate point of these scores was used, and expressed as 45, 55 and 70 beats per minute. It was felt that the preciseness of the measurement was being reduced with no increase in ease of recording. Consequently, in Studies 2 and 3, respiratory rate was measured over a 60 second interval by stopwatch and the actual rate recorded.

Clinical Examinations

Calves were checked twice daily for signs of ill health throughout the trial. For each animal, the clinical demeanour and respiratory rate were recorded and the rectal temperature was measured prior to the morning and evening feeds.

Milk intake records

The quantity of milk offered was recorded for each calf at each feed (twice daily). The volume of any milk not drunk was measured and recorded as a refusal.

The feed refusals were scored as:

0	No refusal, all milk consumed within 30 minutes
1	Milk remaining at 30 minutes but all consumed by next feed (9-12 hours)
2	< 50% milk remaining at next feed (9-12 hours)
3	> 50% milk remaining at next feed (9-12 hours)

Body weight

Calves were weighed at the beginning of the study and immediately prior to necropsy, to the nearest 0.5kg, in a calf weighing crate which had been validated within the three months prior to the study as accurate to at least 0.5kg.

Procedures at necropsy

Description of lesions

At *post mortem* examination, lungs were excised and pneumonic lesions recorded on a standard lung diagram (e.g. consolidation, abcessation, fibrous tagging on the lung surfaces, areas of focal necrosis).

Estimation of percent lung area affected

The percent pneumonic consolidation was estimated using a grid of 100 squares which was placed under the lung and the number of squares underlying affected areas of the lung marked and counted.

Microbiology

Nasopharyngeal swabs were taken from all calves prior to challenge and cultured for *Pasteurella spp.* and *M.bovis.* Lung tissue was taken for identification of bacteria and mycoplasmas present.

Samples of tissue were collected from cranial, diaphragmatic and caudal lung lobes. All the samples were examined for the presence of bacteria and mycoplasmas. All the samples were inoculated on to three media: Colombia blood agar (Oxoid), PPLO agar

(Difco) enriched with 25 percent inactivated horse serum, 7 percent yeast extract, 400 $\mu g.ml^{-1}$ ampicillin, 0.05 percent thallium acetate and 1 percent glucose, and Tween 80 PPLO agar (the same enriched and selective medium with 0.1 percent Tween 80). The bloodplates were evaluated after 24 or 48 hours of incubation at 37°C in a carbon dioxide enriched atmosphere. The mycoplasma plates were evaluated after two days and, if negative, daily until 14 days after inoculation.

Animals (Scientific Procedures) Act 1986

Procedures were carried out under Project licence 90/00780, procedure 14, protocol approved by Dr P.G.G.Darke, HO inspector, Swindon, 18 July 1995. Procedure 14 is classified as moderate. Humane end-points were any calf showing anorexia, marked apathy, dyspnoea, rectal temperature >41°C persisting over a period of 12 hours.

Study 1 (CUK9501) calves:

Study Outline

Ten male Jersey calves were used in Study 1. Calves were fed two litres of milk replacer twice daily at 0700hrs and 1800hrs.

Experimental challenge

Calves were challenged by a trans-tracheal route with *P.haemolytica* serotype A1, isolated from a case of pneumonic bovine pasteurellosis (source: Institute for Animal Health, Compton: B756 from calf lung 1090/IAH 141, originally strain GC1 Gourlay and Houghton, 1985) and *M.bovis* (source: K688 from calf lung 4171/IAH 333, originally from VIC, Sutton Bonnington, 1992) also from a naturally occurring case of calf pneumonia. The inoculum was administered as a

20ml dose being a 10ml suspension in culture medium of each microorganism that contained 2×10^7 colony forming units (cfu) of *P.haemolytica* and 1×10^8 colorimetric colour units (ccu) of *M.bovis.* A 5cm square patch overlying the trachea one third of the way down the neck of each calf was clipped, cleaned and swabbed with surgical spirit. A 5cm 18 gauge needle was inserted directly into the tracheal lumen, a 20ml syringe containing the inoculum was attached and the inoculum introduced. Before and after inoculation, correct positioning of the needle was ascertained by withdrawing the plunger and confirming that air was aspirated into the syringe.

Schedule of study events

Day 1	Arrival of calves in experimental animal house, general health examination, feed with electrolyte glucose solution
Day 2	Start feeding with milk replacer twice daily
Day 8	Take nasopharyngeal swabs all calves and culture for Pasteurella and Mycoplasma
Day 12	Allocate calves to accommodation, weigh all calves
Day 15	Trans-tracheal infection with *P.haemolytica* and *M.bovis*
Day 22	Weigh, euthanase and necropsy calves

RESULTS

Colostrum intake, incidental disease and calf management

Calves weighed between 24 and 31 kg at the beginning of the study. All calves lost weight during the study, but the clinical importance could not be statistically quantified in the absence of a non-infected control group (Table 1). All ten calves experienced diarrhoea and inappetance prior to the start of the trial and were treated with oral kaolin (Kaogel, Parke Davis, Pontypool, UK), a subcutaneous glucose solution (Calvet no.8, Animalcare Ltd, Dunnington, UK) and oral glucose/electrolyte solution (Lectade) was substituted for milk-replacer powder until the calf recovered, which in each case was within 48 hours (by the fourth feed). Rotavirus infection was diagnosed by an Enzyme Linked Immuno-Sorbent Assay (ELISA) antigen capture kit in faecal samples from calves 1, 2, 4, 7 and 8 and this same infection was presumed to be the cause of disease in the other five calves.

Table 1: Start and finish weights of ten calves after experimental infection with *P.haemolytica* and *M.bovis*.

Calf no.	Start weight (kg)	Finish weight (kg)
	Calves euthanased at the end of the study (7 days after infection)	
1	31	28
3	29	29
7	24	21
8	25	23
10	24	20
12	24	22
	Calves euthanased before the end of the study (within 24 hours)	
2	29	29
4	28	27.5
9	28	28
11	28	27.5

Challenge infection

Prechallenge nasopharyngeal swabs taken from all ten calves were negative for *P.haemolytica* and *M.bovis*. The challenge was accomplished satisfactorily in nine of the ten calves. Calf 4 struggled immediately after inoculation and although the inoculum was presumed to have been delivered satisfactorily this could not be ascertained by withdrawal of air from the trachea. The subsequent response of this animal and the pneumonic lesions detected at *post mortem* confirmed the success of the inoculation.

Clinical findings and scores

All calves coughed intermittently (1-2 min duration) following challenge infection.

Clinical signs in the four animals euthanased before the end of the study comprised of pyrexia, inappetance and failure to respond to the presence of the observer (clinical score 3, severe disease). Sternal recumbency, tachypnoea (respiratory rate above 45 breaths per minute) and inappetance (feed refusal score of 3 at last feed) were evident in three of these animals within seven hours of challenge. Calf numbers 2, 9 and 11 were euthanased at this stage having reached the moderate severity band, as defined by the project licence, the fourth animal (calf no.4) that was not recumbent was left for a further 11 hours before being killed, by which stage it was recumbent, tachypnoeic and presented with clinical demeanour score 3 ('severe disease'). Abnormal clinical signs were not evident in five calves for the duration of the trial. Calf no.12, for which a clinical score of 3 was recorded, showed inappetance on three occasions but this was unrelated to challenge since inappetance had been recorded prior to infection and was related to an episode of enteritis and diarrhoea. This calf was clinically healthy by the time of euthanasia at the end of the study.

Individual animal rectal temperatures, clinical demeanour scores and respiratory rates are given in Table 2. Feed refusal scores did not yield any useful data because of the peracute nature of the experimental infection. Calves progressed from having a full appetite and showing no clinical signs to a clinical condition that required euthanasia within a 12 hour period. The last feed refusal score therefore often did not relate to the clinical condition. However, all calves which required euthanasia and all calves which showed increased demeanour scores or a rectal temperature at or above 39.7°C had increased feed refusal scores. Due to the small number of calves (4) in this subcategory it would be misleading to attempt to correlate this data. Table 3 provides a summary of each parameter recorded for each calf in this study. Although clinical score data is ordinal, the numeric scale does not reflect a linear disease progression, only a progression in easily recognisable signs. Therefore, non-parametric statistical tests based on the ranks of the data values rather than the actual numbers recorded were used. Graphical illustrations of the relationship between clinical score and rectal temperature (Figure 1) and respiratory score and rectal temperature (Figure 2) portray the relationships which were statistically tested for strength of correlation (Table 18). A two-way table (Table 4) shows the relationship between the two clinical scoring systems used (i.e. clinical [demeanour] and respiratory scores).

Table 2: Clinical scores, % pneumonic consolidation and recovery of *P.haemolytica and M.bovis* from lungs of calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*

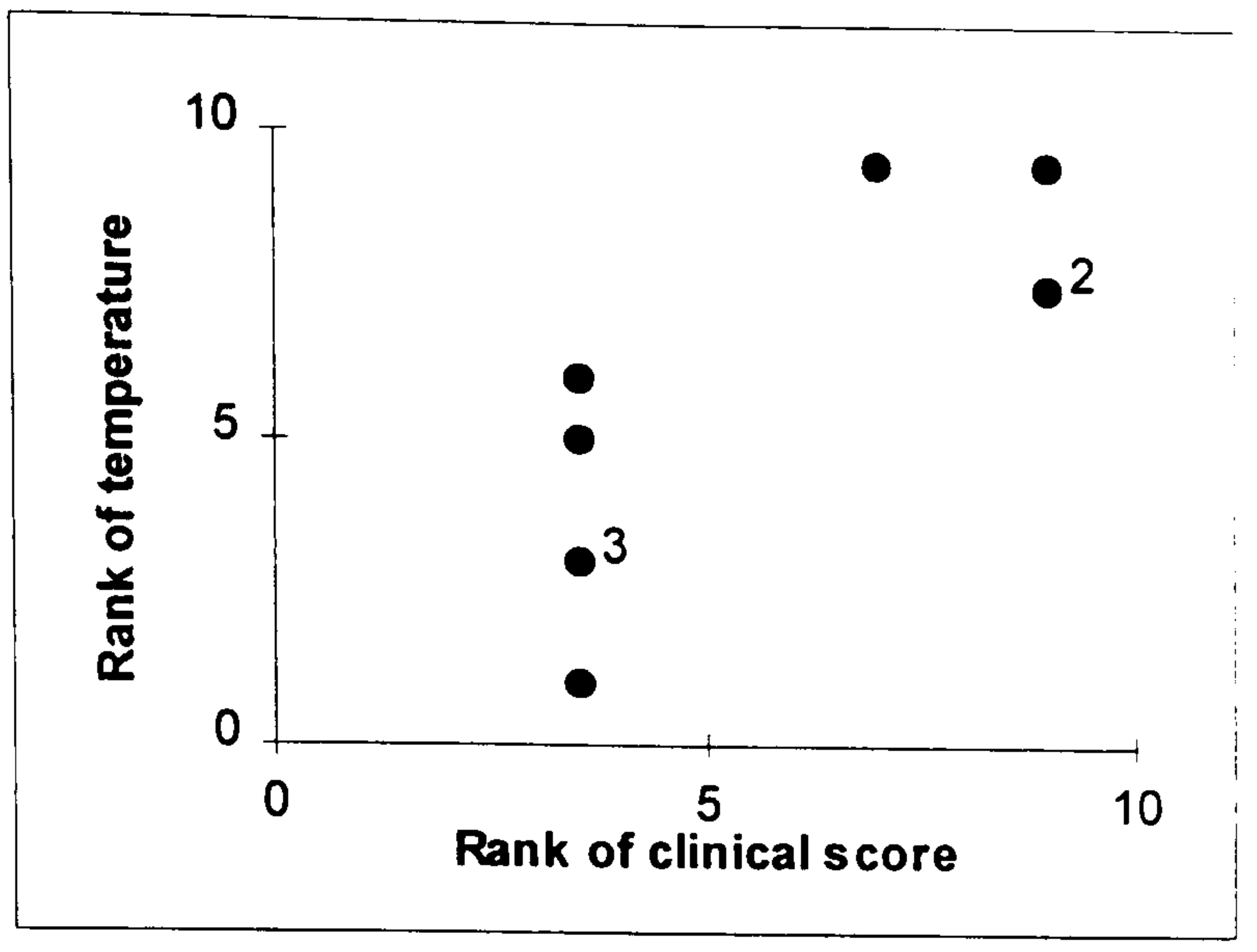
calf no.	terminal demeanour score	% lung consolidation	reisolation from lung of: *P.haemolytica*	*Mbovis*
		Animals surviving to end of study		
1	0	2	-	-
3	0	3	-	-
7	0	3	-	-
8	0	4	-	-
10	0	1	-	-
12	0	4	-	-
mean		**2.8 (SD = 1.6)**		
		Animals euthanased during course of study		
2	3	7	+	+
4	3	16	+	+
9	3	7	+	-
11	3	7	+	-
mean		**9.25 (SD= 3.9)**		

SD = Standard deviation
\+ = Positive culture of *P.haemolytica or M.bovis*

Table 3: Terminal clinical scores and *post mortem* pathological measures of disease severity of ten calves experimentally infected with a combined experimental infection of *Pasteurella haemolytica* A1 and *Mycoplasma bovis*

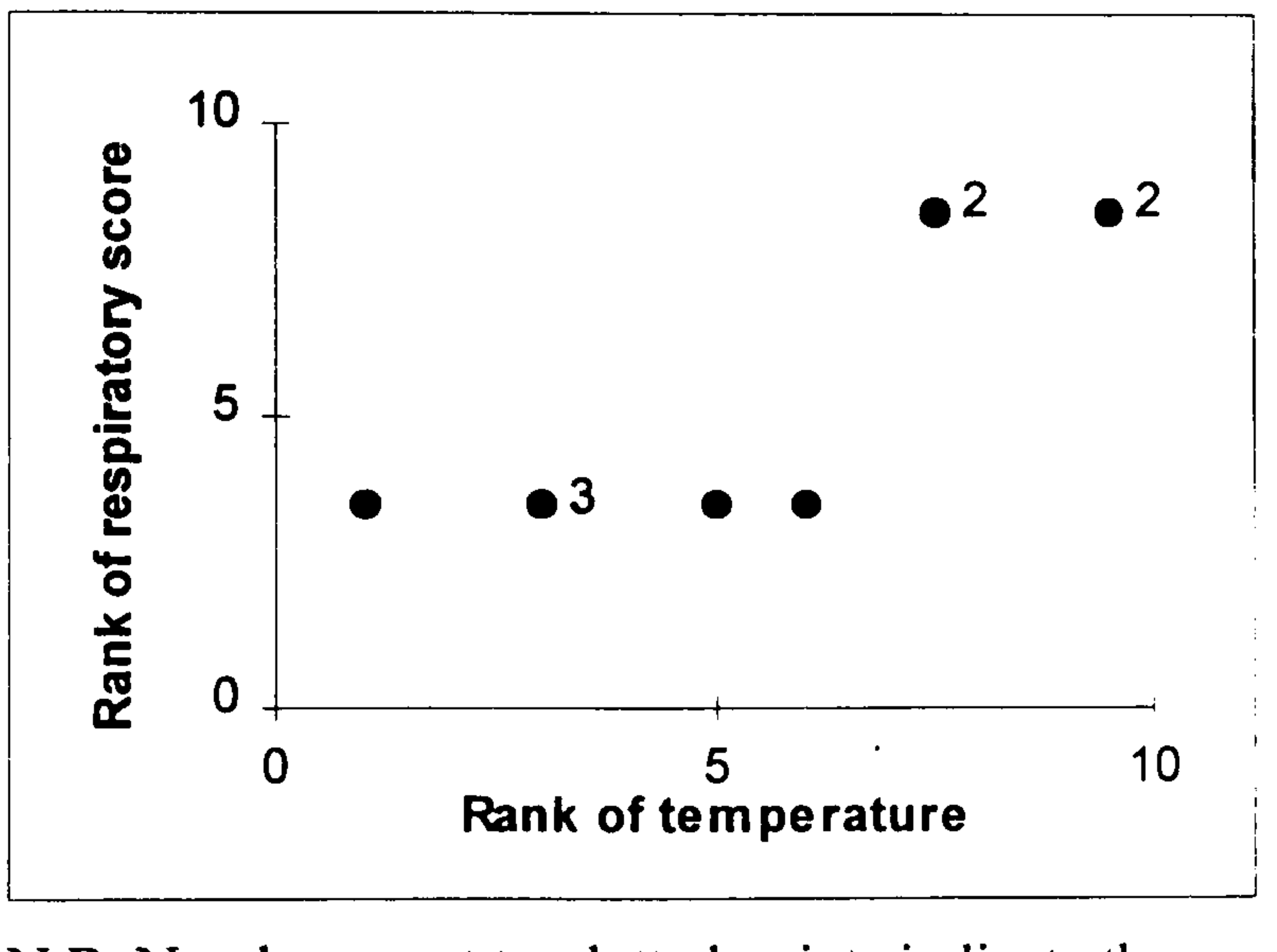
CUK9501 Terminal Score	Identity number of calf	Terminal rectal temperature (°C)	Terminal respiratory rate (breaths/minute)	Percent lung consolidation	Bacterial reisolation
0	12	38.5	45	4	no
0	10	38.6	45	1	no
0	8	38.2	45	4	no
0	7	38.2	45	3	no
0	3	38.1	45	3	no
0	1	38.2	45	2	no
2	9	39.8	70	7	yes
3	2	39.8	70	7	yes
3	4	39.7	70	16	yes
3	11	39.7	70	7	yes

Figure 1: Graphical relationship between ranked rectal temperature and clinical score data for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*



N.B. Numbers next to plotted points indicate the number of identical values

Figure 2: Graphical relationship between ranked respiratory score and rectal temperature data for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*



N.B. Numbers next to plotted points indicate the number of identical values

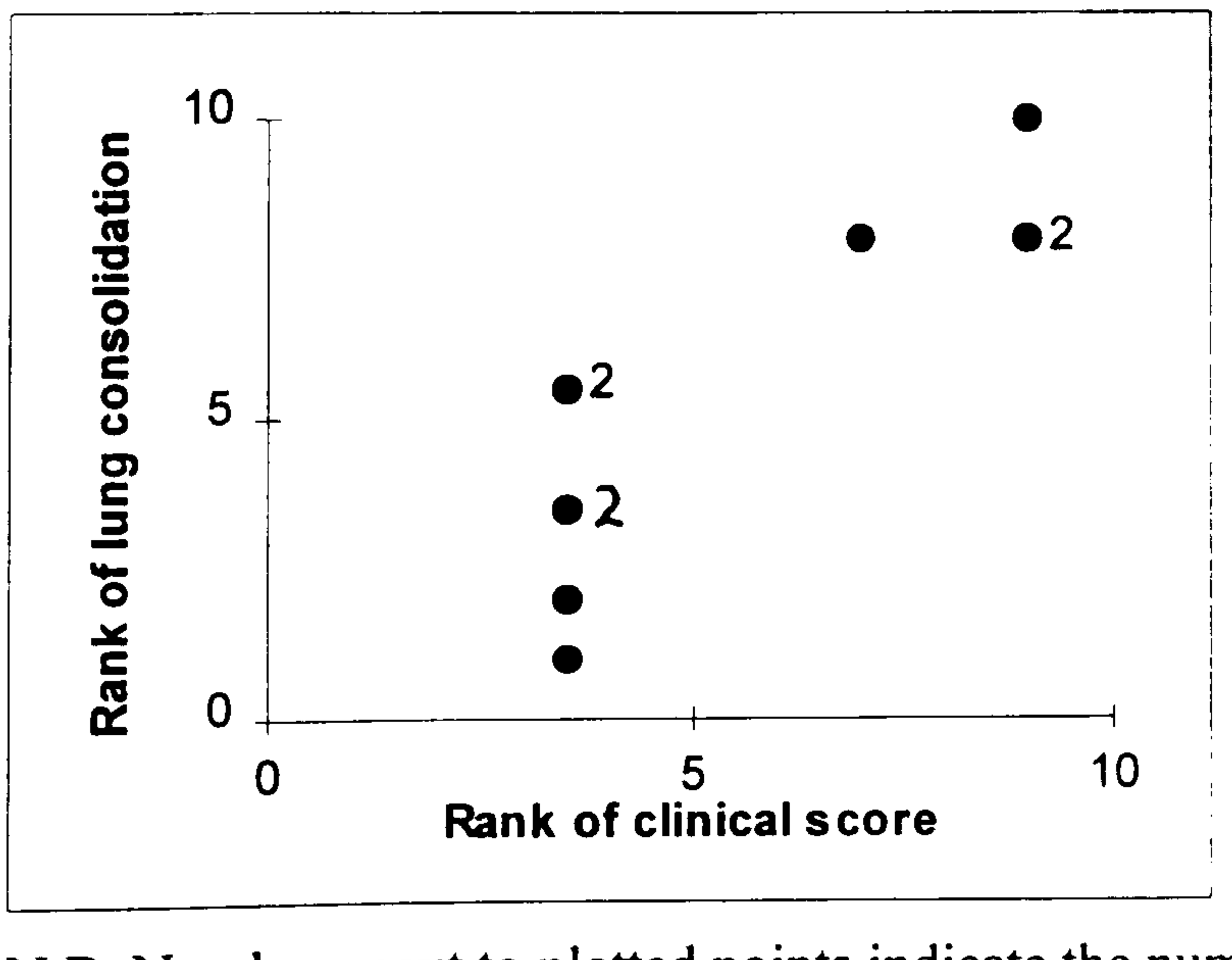
Table 4: Number of animals with minimum and maximum respiratory scores at each clinical score for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*

Number of animals	Clinical score 0	2	3
Respiratory score			
0	6	0	0
3	0	1	3

Gross pathological findings

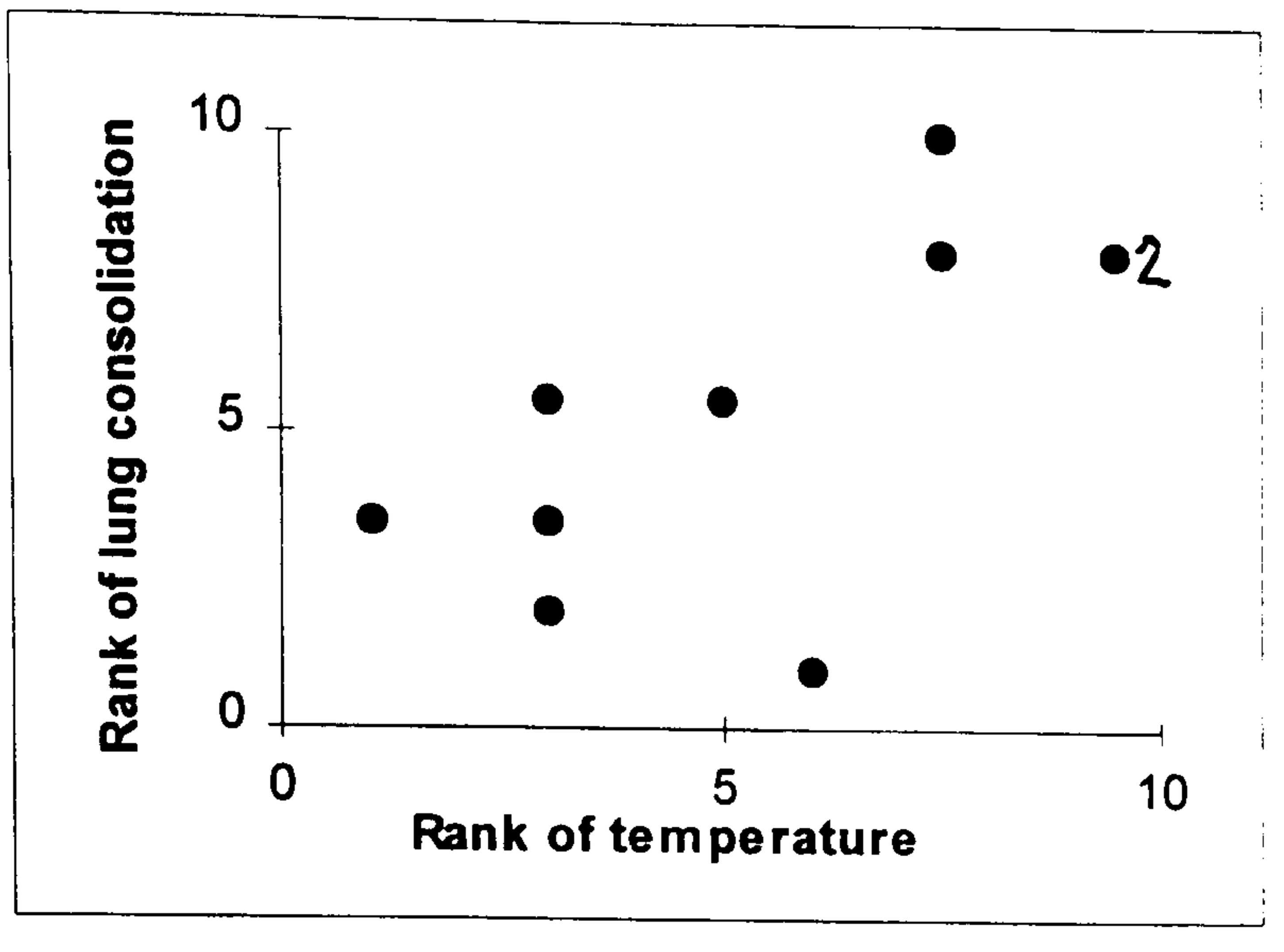
The mean pneumonic consolidation score for the six calves euthanased seven days post challenge was 3% [to 1 significant figure] (Standard deviation = 1.6), mean score for the four calves killed 8-19 hours post-challenge was 9% [to 1 significant figure] (Standard deviation = 3.9) (Table 2). These scores are not comparable because of the different duration of infection. The character of the lesions between the two groups reflect this incomparability. The three days' duration pneumonic lesions showed red/grey hepatisation and a stippled distribution. The stellate nature of the lesions indicated resolution, in contrast, the 7 hour-old lesions were red hepatised, swollen and slightly oedematous. This latter type of lesion was most marked and extensive in calf no.4 that was killed 18 hours post-challenge. Individual pneumonic lesions were recorded on standard lung diagrams. Figures 3, 4 and 5 illustrate the relationship between lung consolidation data and clinical score, rectal temperature and respiratory rate, respectively.

Figure 3: Graphical relationship between ranked lung consolidation and rank of clinical score for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*



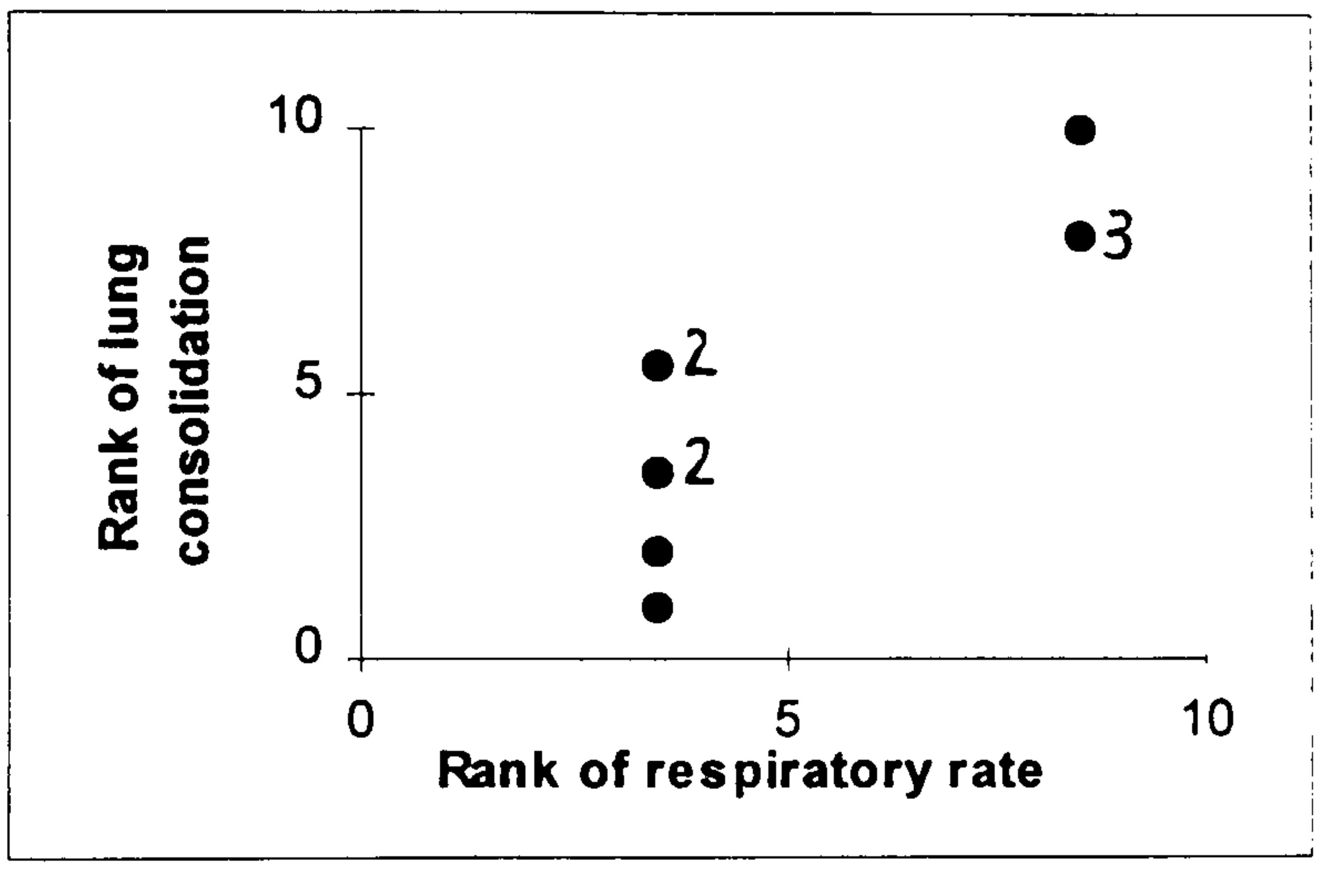
N.B. Numbers next to plotted points indicate the number of identical values

Figure 4: Graphical relationship between rank of lung consolidation and rank of rectal temperature for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*



N.B. Numbers next to plotted points indicate the number of identical values

Figure 5: Graphical relationship between rank of lung consolidation and ranked respiratory rate data for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*



N.B. Numbers next to plotted points indicate the number of identical values

Microbiological findings

P.haemolytica was re-isolated from all four of the calves euthanased before the end of the study and *M.bovis* was also isolated from two of these calves (Table 2). Neither microorganism was isolated from the six calves which survived to the end of the study. The relationship between the clinical score and the bacteriology results is expressed in Table 5, and between respiratory score and bacteriology in

Table 6. Table 2 has already shown the relationship between the bacteriology results and the lung consolidation data obtained at *post mortem* examination.

Table 5: Number of calves at each clinical score from which *P.haemolytica* was successfully re-isolated for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*

Number of animals	Clinical score 0	2	3
Bacterial reisolation			
no	6	0	0
yes	0	1	3

Table 6: Number of successful bacterial isolations from calves with maximum and minimum respiratory scores for ten calves experimentally infected with a combination of *Mycoplasma bovis* and *Pasteurella haemolytica*

Number of animals	Respiratory score 0	3
Bacterial reisolation		
no	6	0
yes	0	4

A summary of results for Study 1 is given in Table 7. Table 8 illustrates the system used for the allocation of ranks, whereby each parameter was graded on a scale of 1-10. This facilitated cross-group statistical analysis and graphical illustration.

Table 7: Summary of relationship between terminal clinical scores and mean clinical and pathological data of ten calves experimentally infected with a combined experimental infection of *Pasteurella haemolytica* A1 and *Mycoplasma bovis*

Score	Number of animals	Mean rectal temperature (°C)	Mean respiratory rate (breaths/minute)	Mean % lung consolidation	% Bacterial reisolation
0	6	38.3	45	3	0
1	-	-	-	-	-
2	1	39.8	70	7	100
3	3	39.8	70	10	100

Table 8: Ranked data from Study 1, for ten calves experimentally infected with *Pasteurella haemolytica* and *Mycoplasma bovis*

CUK9501 Clinical score	Calf no.	Rectal Temp.	Resp. score	Lung consolidation (%)	Bacteriol. reisolation	RANKS Clinical score	RANKS Rectal Temp.	RANKS Resp. score	RANKS Lung consolidation (%)	RANKS Bacteriol. reisolation
0	12	38.5	0	4	no	3.5	5	3.5	5.5	3.5
0	10	38.6	0	1	no	3.5	6	3.5	1	3.5
0	8	38.2	0	4	no	3.5	3	3.5	5.5	3.5
0	7	38.2	0	3	no	3.5	3	3.5	3.5	3.5
0	3	38.1	0	3	no	3.5	1	3.5	3.5	3.5
0	1	38.2	0	2	no	3.5	3	3.5	2	3.5
2	9	39.8	3	7	yes	7	9.5	8.5	8	8.5
3	2	39.8	3	7	yes	9	9.5	8.5	8	8.5
3	4	39.7	3	16	yes	9	7.5	8.5	10	8.5
3	11	39.7	3	7	yes	9	7.5	8.5	8	8.5

Discussion (Study 1)

The difference in clinical demeanour between the calves which required euthanasia before the end of the study and those which remained until the end of the experiment is illustrated by the terminal clinical demeanor scores (0 or 3, Table 2). A difference was also seen in the mean pneumonic consolidation scores (2.8 versus 9.25, Table 2) but these are not comparable since the interval to euthanasia, and therefore *post mortem* examination, for the two groups was different. However, differences were noted in the character of the macroscopic pneumonic lesions. The lesions in the calves killed only seven to 18 hours post-challenge were red, hepatised, oedematous and slightly raised whereas in the remaining calves, the stippled character of the lesions, the red/grey hepatisation and lack of oedema indicated a resolving lesion. The failure to reisolate either *P.haemolytica* or *M.bovis* from these lesions in the calves which survived to the end of the study supports the suggestion that the lesions were resolving.

The experimental infection model appeared to work well in so far as all animals showed either clinical or pathological evidence of the experimental challenge. *P.haemolytica* was isolated from all four of the calves euthanased early in the study, in addition to *M.bovis* from two of these calves.

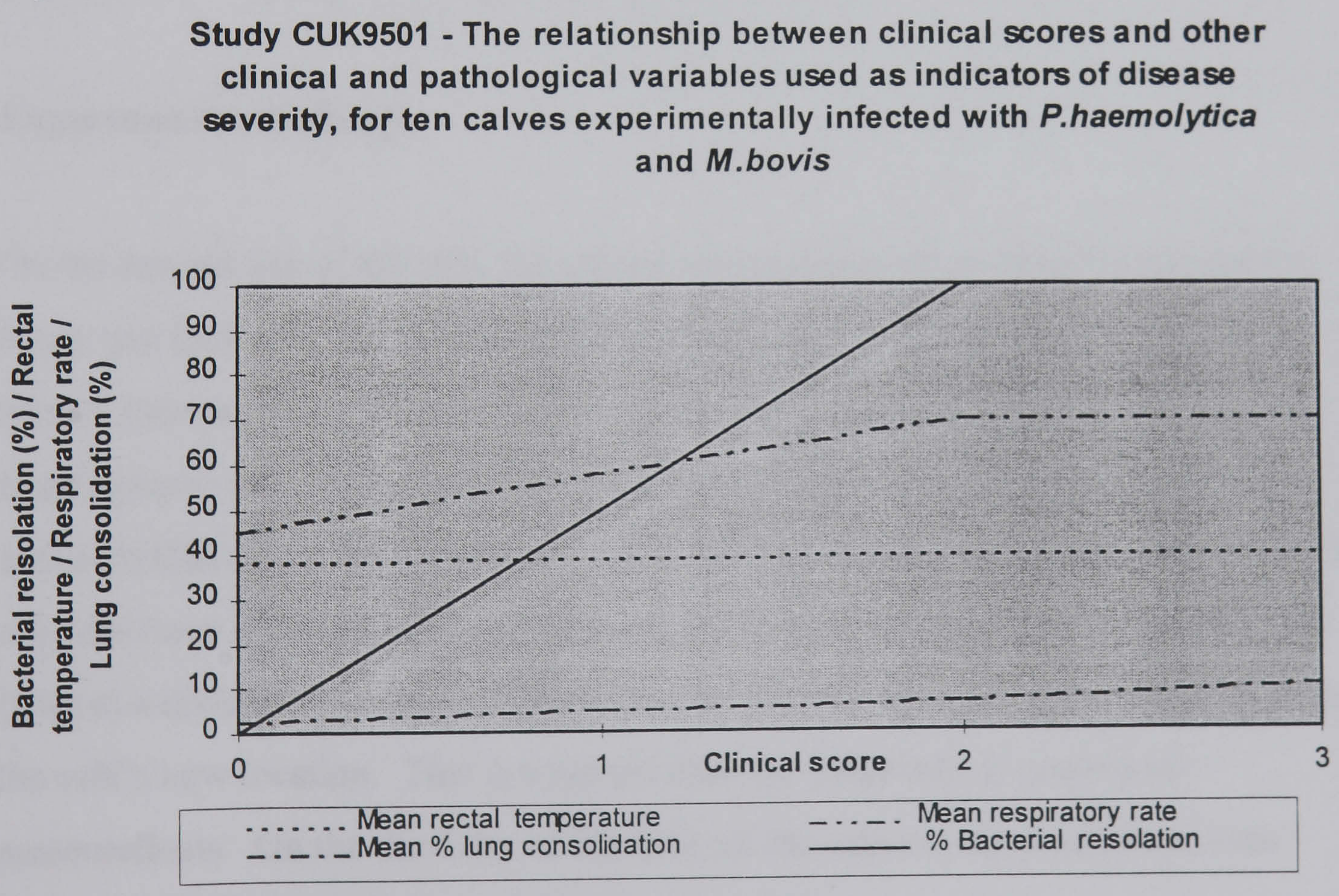
The drop in bodyweight seen in five of the six calves which survived to the end of the experiment was not statistically significant, though, it is worth noting that over a seven day period no calf gained weight. The weight of the calves euthanased early in the study did not alter much in the short period that they were on the study. Whilst weight loss could be due to inappetance and increased tissue catabolism during disease, it could also relate to a reduction in gut fill from the pre-infection level when calves had recently received a two litre feed and the final weighing which was up to 12 hours after the last feed. The diarrhoea due to the rotavirus infection would also contribute towards impaired growth. Furthermore, some calves had been inappetant at the last feed.

Respiratory rate , percent lung consolidation and bacterial reisolation increased as clinical score increased (Table 7). A comparison between calves with a terminal score of 0 and those with a terminal clinical score of 2 or 3 showed a significant increase in each of the above parameters (Kruskal-Wallis test, p=0.05). In this study, there seemed to be a rectal temperature threshold above which respiratory rate, lung consolidation and bacterial reisolation were significantly increased. This threshold temperature appeared to be above 38.6°C and less than 39.7°C (Table 3). This is comparable with thresholds cited as indicative of respiratory disease by Bols and de Kruif (1994), Lauridsen *et al.* (1994) and Scott (1994). The scale of the graph (Figure 7) does not clearly show the increase in mean rectal temperature by 1.5°C above normal body temperature as the condition of calves deteriorated from a clinical score of 0 to 3 (Table 7).

An increase in clinical score does not reflect uniform linear increases in other clinical signs such as respiratory rate or rectal temperature which increased more rapidly in “subdued” or “apathetic” calves initially (i.e. between scores 0-2) than for calves with higher clinical scores (scores 2 and 3) or for lung consolidation (although this later measure was within a narrow range of 1-7% and 7-16% in nine of the calves (Figure 7). More data are needed before firm conclusions can be drawn from this study.

Overall, an increase in clinical score did appear to correlate to increased lung consolidation, and in turn, as might be expected, to both increased respiratory rate and isolation of the infecting organism. The inflammatory changes associated with lung damage induced by the pathogen would be expected to cause pyrexia. The alternative pathway of the complement cascade is activated by the presence of lipo-polysaccharide in the bacterial membrane, attracting phagocytes. Necrosis of neutrophils, heightened by leucotoxin release, triggers additional release of cytokines which not only cause pyrexia but are chemo-attractant to leukocytes and increase pulmonary vascular permeability which augments cellular infiltration. Consequently, along with cellular infiltration, oedema and consolidation of the lung, the rectal temperature also rises. Formal statistical analysis of the strength of these relationships is discussed at the end of this chapter in conjunction with the results from other studies.

Figure 6:



Study 2 - (CBE9501) calves:

Study Outline

Sixteen male Friesian-Holstein calves with a mean body weight of 41.4 (± 1.7) kg (range 36-44 kg) were experimentally infected intra-tracheally with *Pasteurella haemolytica* type A1 (LPB 1419). The calves were fed three litres of milk replacer twice daily at 0900hrs and 1800 hrs.

Schedule of study events

Day 1	Arrival of calves in experimental animal house, general health examination of calves, feed glucose/electrolyte solution, weigh calves
Day 2	Transportation of calves in closed trucks, start feeding milk replacer twice daily
Day 3	Deep endo-bronchial infection with *P.haemolytica* A1
Day 10	Weigh, euthanase and necropsy calves

Experimental challenge

On the second day of the trial, the calves were transported in closed trucks for two hours and then returned to the animal accommodation. This was done to simulate the typical transportation stress experienced by young calves when being transported large distances across Europe from their herd of origin to calf raising units. Upon arrival at these units, they typically encounter new infection challenges for which they may not have adequate passively derived antibody protection from colostrum from cows at a different location which had not been exposed to the microbial population at the calf's new location. This is a typical time for outbreaks of respiratory pasteurellosis. On the third day of the trial, all the calves were inoculated intra-tracheally with *P. haemolytica* type A1.

Experimental infection procedure

A first-pass culture of *P. haemolytica* type A1 was inoculated into 10 ml quantities of brain-heart-infusion broth (Oxoid), enriched with 5 percent fetal calf serum and incubated at 37°C in a shaking water bath for six hours. The approximate bacterial count of each broth after six hours of incubation was 1×10^9 cfu ml^{-1}. A polyethylene catheter ('Intramedic', VEL) was inserted through the right nostril and advanced into the trachea until it was 5 cm proximal to the bifurcation. The single dose inoculum consisted of 5 ml of the six-hour culture of *P. haemolytica* type A1 diluted with 5 ml of sterile 0.9 percent sodium chloride.

RESULTS

As with the previous study, percent lung consolidation and respiratory rate increased with worsening clinical score (Tables 9 and 10). The rate of these changes is not linearly proportional. Bacterial isolation showed the same trend, however, a failure of isolation in the single calf with a score of 2 accounts for the 'saw tooth' shape of the graph (Figure 13).

Table 9:

T5CCBE					
Relationship between clinical scores and rectal temperature immediately prior to *post mortem*, and % lung consolidation and bacterial re-isolation from lungs during necropsy, for sixteen calves endo-bronchially inoculated with *P.haemolytica* Type A1					
Clinical score	**Identity no. of calf**	**Mean rectal temperature °C**	**Respiratory rate breaths/minute**	**% lung consolidation**	**Bacteriological reisolation**
0	5575	39.4	24	0	no
0	9551	39	32	30	no
0	3597	39	28	0	no
0	5071	39.4	24	0	no
0	7497	39.5	36	30	no
0	2382	38.6	28	0	no
1	7144	38.9	52	15	all lobes
1	6639	40.8	48	30	no
2	2772	41	60	90	no
3	2533	40.2	96	80	all lobes
3	9868	40.4	64	80	all lobes
3	6720	40.3	84	25	all lobes
3	5678	40.6	96	80	all lobes
3	8322	38	52	30	4/6 lobes
3	4627	39.8	40	10	all lobes
3	4624	40.1	72	80	negative

Table 10: Summary of relationship between clinical scores and means of other variables for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1

Score	Number of animals	Mean rectal temperature	Mean respiratory rate	Mean % lung consolidation	% Bacterial reisolation
0	6	39.2	29	10	0
1	2	39.9	50	22	50
2*	1	(41)	(60)	(90)	(0)
3	7	39.9	72	55	85

* N.B. only one animal had a score of 2 so mean data may be misleading

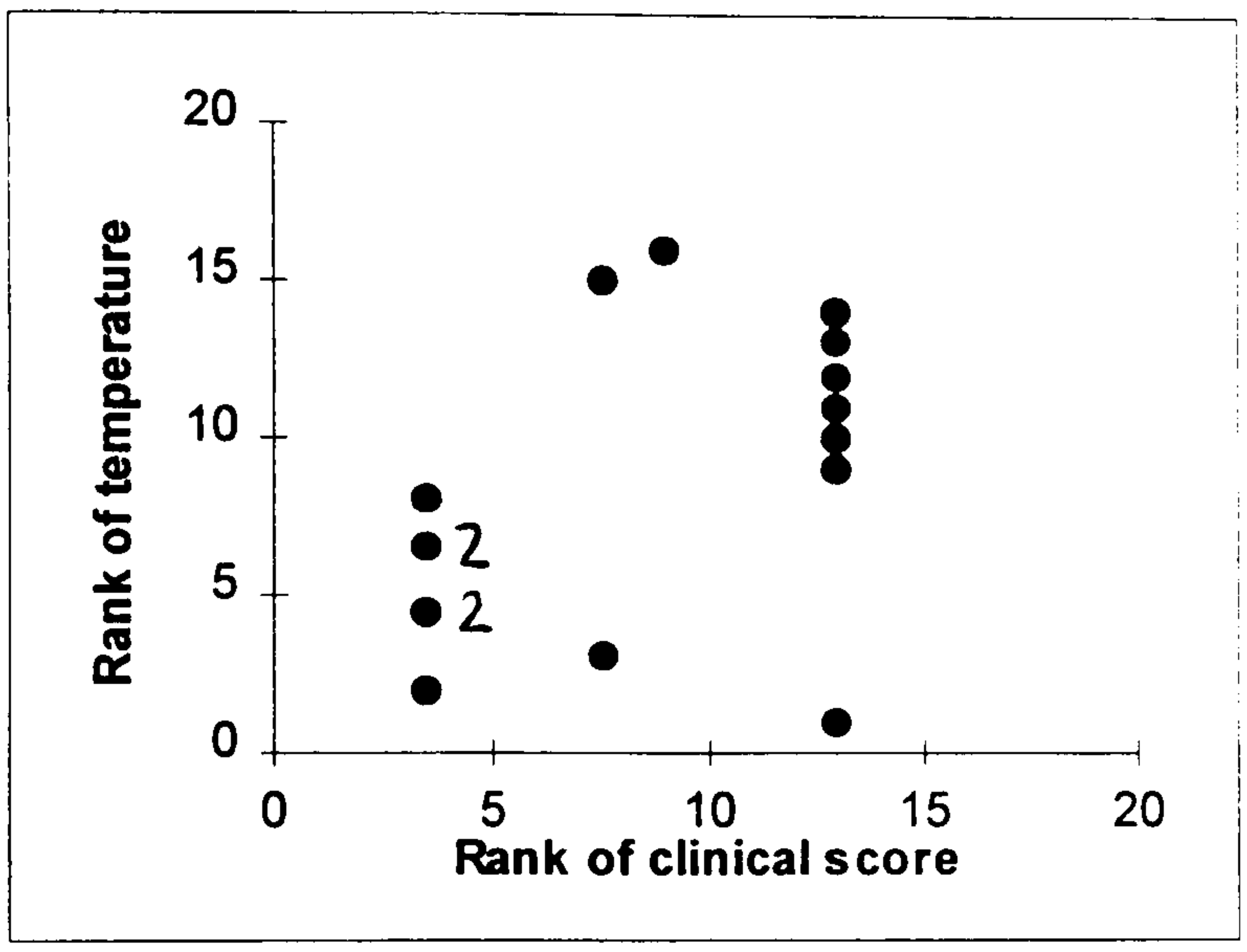
Mortality

Eight calves were euthanased before the end of the trial, four of them within 24 hours of inoculation.

Rectal temperature

Eight hours after infection, four calves showed pyrexia (40.4 ± 0.2°C). The mean rectal temperature of the other calves showed no increase. The relationship between ranked rectal temperature and clinical data is illustrated in Figure 7. Apart from two calves (calves 7144 and 8322), the other eight calves with a clinical demeanour score of one or more had a rectal temperature above 39.7°C. None of the six calves with a clinical demeanour score of 0 had a rectal temperature above 39.5°C.

Figure 7: Graphical relationship between rectal temperature and ranked clinical data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1

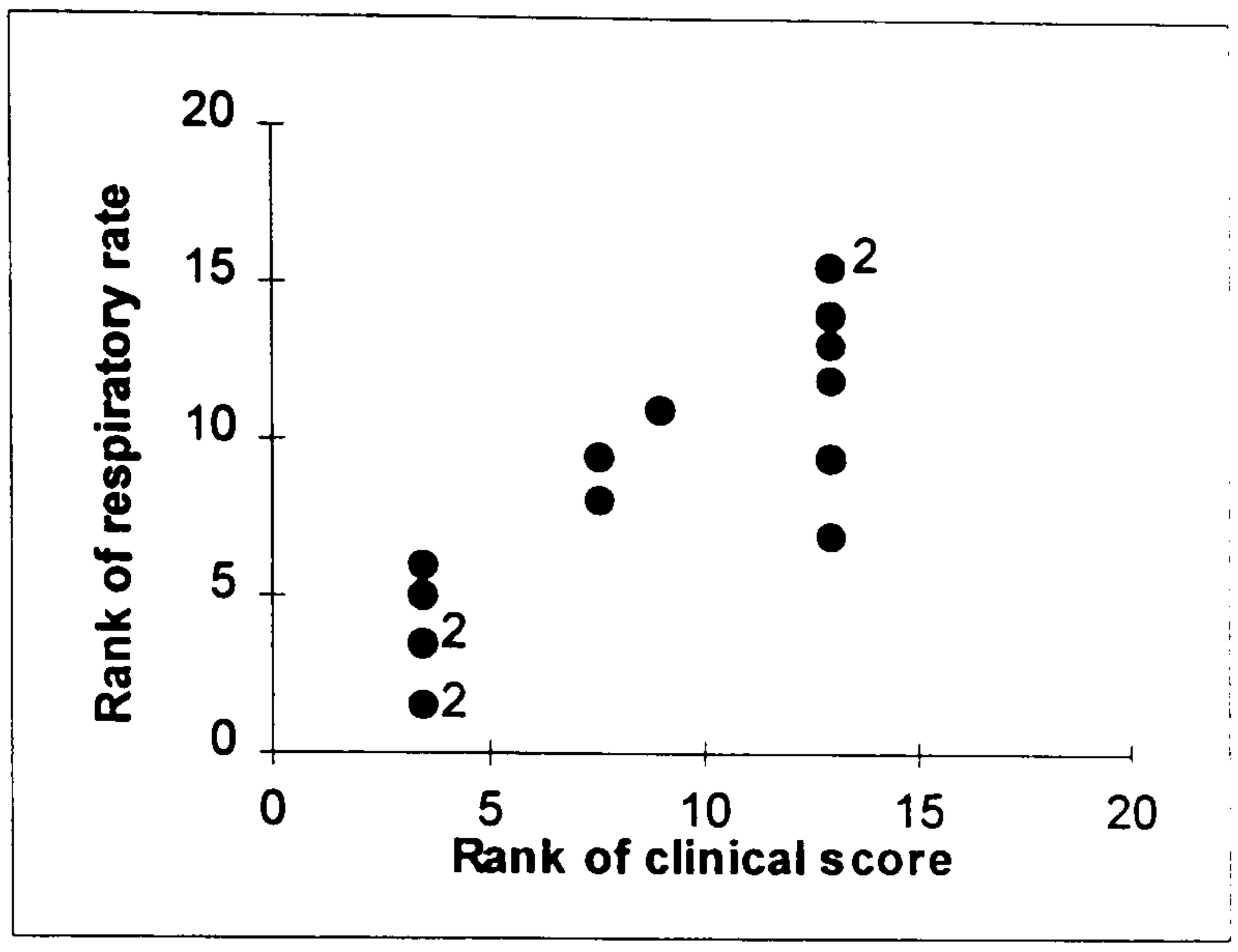


N.B. Numbers next to plotted points indicate the number of identical values

Respiratory rate

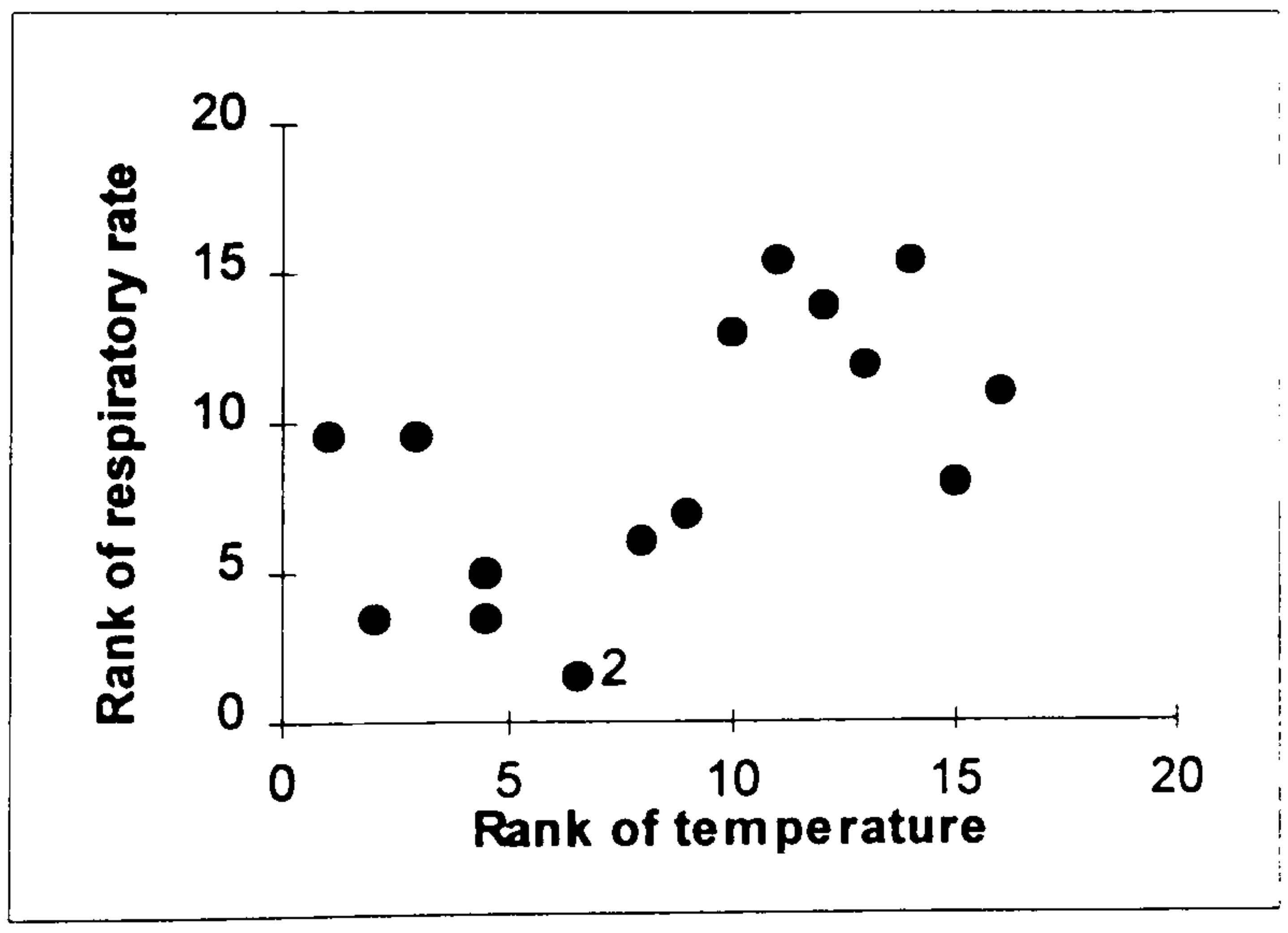
Eight hours after the experimental infection, all the calves were tachypnoeic (range 52-105). The respiratory rate of eight calves remained elevated until they were euthanased for welfare reasons. The mean respiratory rate of the other eight calves had returned to normal on the second day after inoculation. In four of these calves, there was a relapse of signs and the mean respiratory rate increased again on the seventh day after the inoculation and remained elevated (above 60 breaths per minute) until the end of the study. The relationship between respiratory rate and clinical score, and respiratory rate and rectal temperature is illustrated in Figures 8 and 9, respectively.

Figure 8: Graphical relationship between ranked respiratory rate and ranked clinical score data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1



N.B. Numbers next to plotted points indicate the number of identical values

Figure 9: Graphical relationship between ranked respiratory rate and ranked rectal temperature data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1



N.B. Numbers next to plotted points indicate the number of identical values

Respiratory type

The euthanased calves were all tachypnoeic (> 45 breaths per minute) and were also severely dyspnoeic eight hours after the infection. Their respiratory type remained very laborious until they were euthanased. In the other calves, the respiratory type was tachypnoeic and some showed signs of dyspnoea eight hours after the inoculation but returned to normal within 24 hours of the inoculation. Some of these calves coughed and purulent nasal discharges were apparent after the infection but there were no other clinical signs. In eight calves, the clinical score increased again nine days after the infection and remained increased until the end of the trial.

Appetite

All the calves had a severely reduced appetite eight hours after the infection (appetite scores 2 and 3). The appetite of the calves which showed a relapse in clinical signs was more reduced (score 3) than calves which made a full recovery from the sixth day after the inoculation until the end of the trial (score 2). All calves which were euthanased before the end of the study had feed refusal scores of 3. No calves exhibited appetite score 1. If all of the milk replacer was not drunk within a short time of feeding (< 30 minutes), the calves did not show any further interest in drinking it. This is probably partly due to the fall in temperature of the milk replacer solution from 35-40°C at feeding time to room temperature, which makes it less appetising for the calf.

Post mortem examination

Macroscopic pathology

Calves euthanased before the end of the study

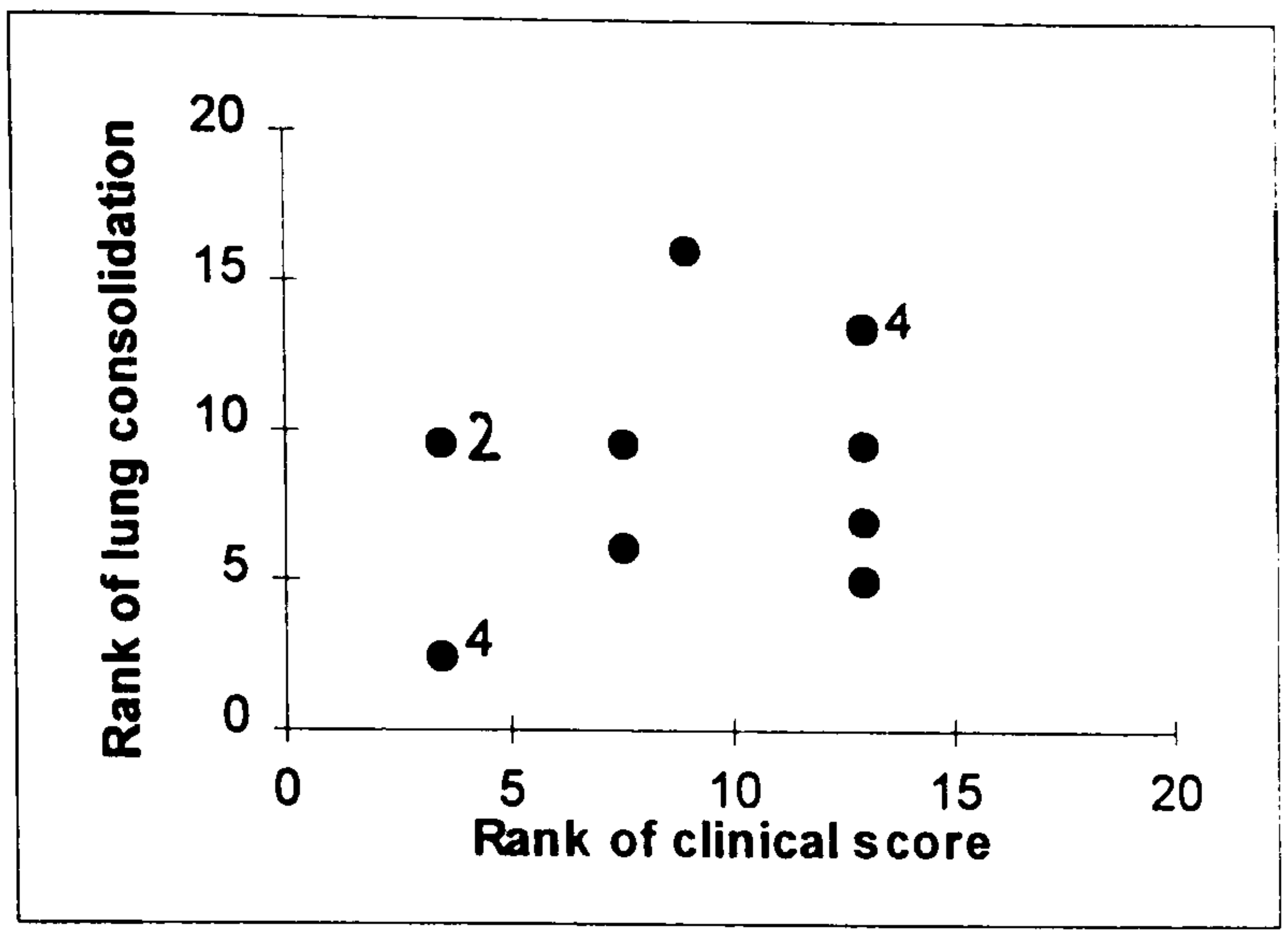
In five of the calves, the lung lesions were severe and abundant and involved all lung lobes, 80-90 percent of the surface area of the lungs showed changes. The lesions were characterized by red and grey hepatisation, oedema, necrosis and fibrosis. The cranial and middle lung lobes were most severely affected. The caudal lung lobes were also affected but to a lesser extent.

One calf which was euthanased within 24 hours of inoculation had only moderate lung lesions, involving 15 percent of the surface area of the lungs. The two calves, euthanased on Days 4 and 6, had only moderate lung lesions compromising 10 and 30 percent of the lung area but a severe catarrhal enteritis.

Calves surviving until the end of the study

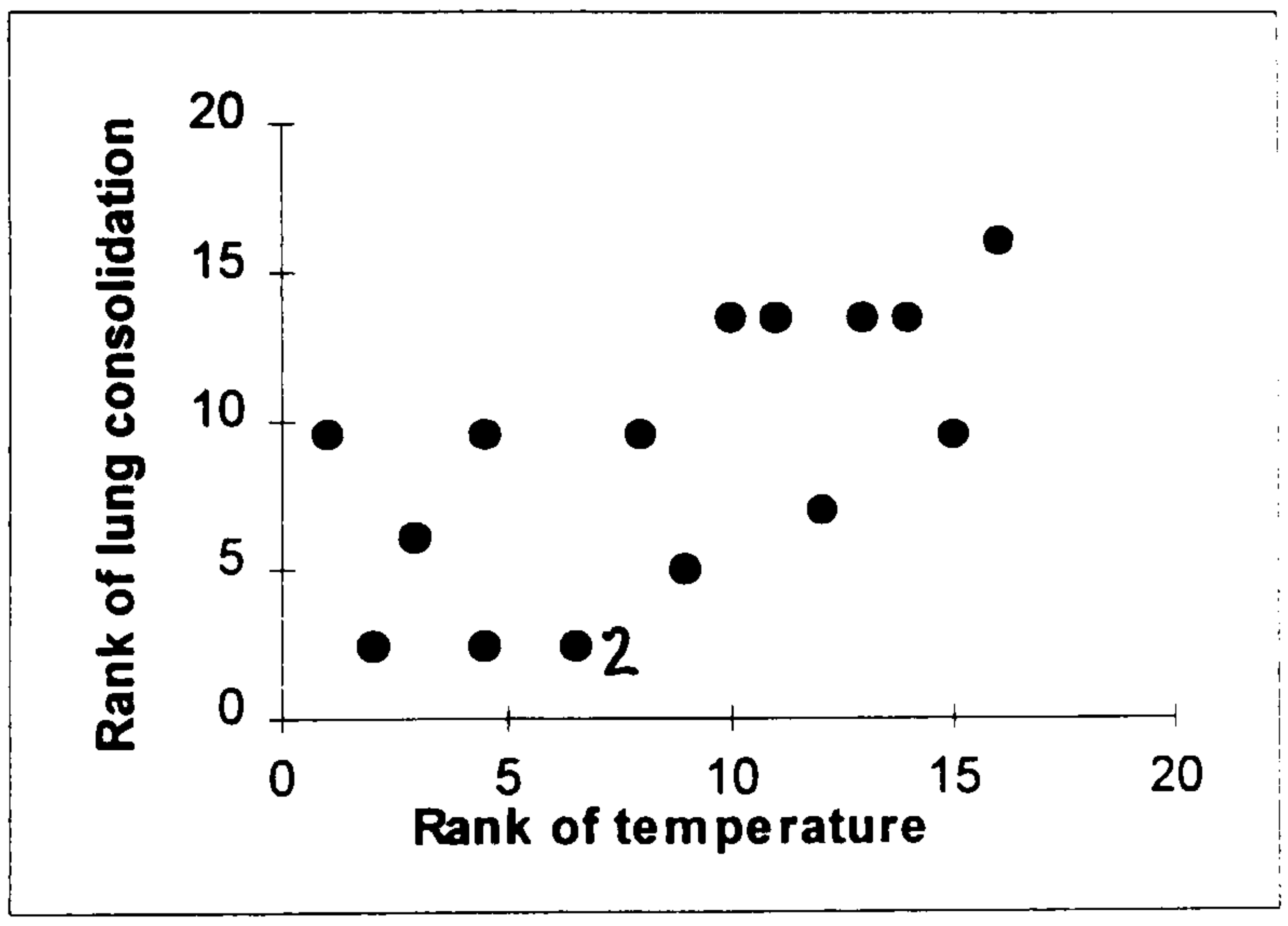
Three of the eight calves which were examined at the end of the study had no lung lesions. Two had only moderate lung lesions involving 30 percent or less of the lung area. In the remaining three calves (Table 9), moderate to abundant lung lesions were evident. In one of them, less than 30 percent of the lungs were affected, presenting red and grey hepatisation in the right apical and middle lung lobes. Figures 10,11 and 12 illustrate the relationship between lung consolidation and clinical score, rectal temperature and respiratory rate respectively.

Figure 10: Graphical relationship between ranked lung consolidation and ranked clinical score data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1



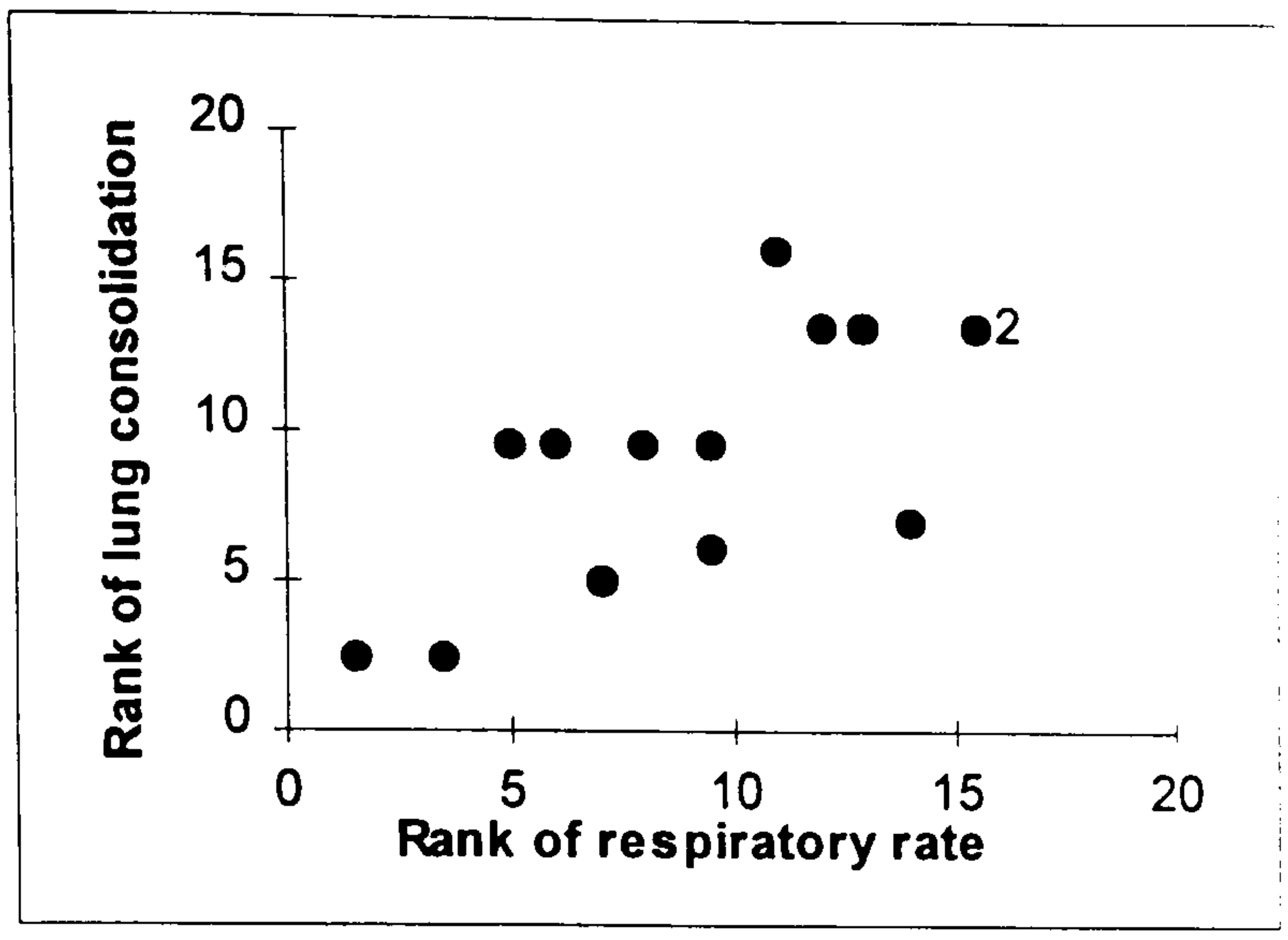
N.B. Numbers next to plotted points indicate the number of identical values

Figure 11: Graphical relationship between ranked lung consolidation and ranked rectal temperature data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1



N.B. Numbers next to plotted points indicate the number of identical values

Figure 12: Graphical relationship between ranked lung consolidation and ranked respiratory rate data for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1



N.B. Numbers next to plotted points indicate the number of identical values

Bacteriology

Calves euthanased during the study

P. haemolytica type A1 was isolated from all the lung samples of the seven of the eight calves euthanased during the experiment. A haemolytic *E. coli* (VTEC) was isolated from the jejunum, colon and mesenteric lymph nodes of a calf euthanased on day 9 of the study. No *P.haemolytica* was reisolated from calf 2772, euthanased during the study.

Calves euthansed at the end of the study

No bacteria were isolated from any of the lung samples. The relationship between clinical score and bacteriological reisolation is illustrated in Table 11.

Table 11: Number of animals from which *P.haemolytica* was successfully reisolated at each clinical score for sixteen calves experimentally infected with *Pasteurella haemolytica* Type A1

number of animals	Clinical score 0	Clinical score 1 or 2	Clinical score 3
Bacterial reisolation			
no	6	2	1
yes	0	1	6

Table 12 provides the individual animal data before infection and at the last measurement prior to necropsy. Table 13 shows the terminal clinical and pathological data and ranked data which was used to portray the gradations in measured variables on the same scale to enable the calculation of correlation coefficients between each combination of clinical and pathological measures and their comparison.

Table 12: Individual animal data before infection and at last measurement prior to necropsy (CBE9501) for calves experimentally infected with *Pasteurella haemolytica* Type A1

CBE9501	Before infection			Terminal measurements prior to necropsy			
Calf No	Rectal Temp.	Resp. rate	Mean clinical score	Rectal Temperature	Respiratory rate	Mean clinical score	Lung consolidation %
9868	38.9	29	0	40.4	64	1	80
5678	39.3	33	0	40.4	92	2.5	80
6720	39.2	32	0.1	40.3	84	3	25
2533	39.9	34	0.8	40.2	90	3	80
2772	39.7	35	0	39.5	46	0	90
7497	38.7	28	0	39	30	0	30
4624	39.1	39	0	39.6	59	1	80
2382	39.1	32	0	38.9	45	0	0
8322	38.9	31	0	39.1	36	1	30
4627	38.8	36	0	38.9	35	1	10
5575	39.1	28	0	39	29	0	0
9551	39.1	32	0	39.3	49	0	30
7114	39.3	32	0	38.9	52	0	15
3597	38.6	31	0	39.1	34	1	0
6639	38.8	30	0	39.3	39	0	30
5071	39.4	32	0	39.2	33	0	0

Table 13: Ranked data for study 2 of calves experimentally infected with *P.haemolytica* A1

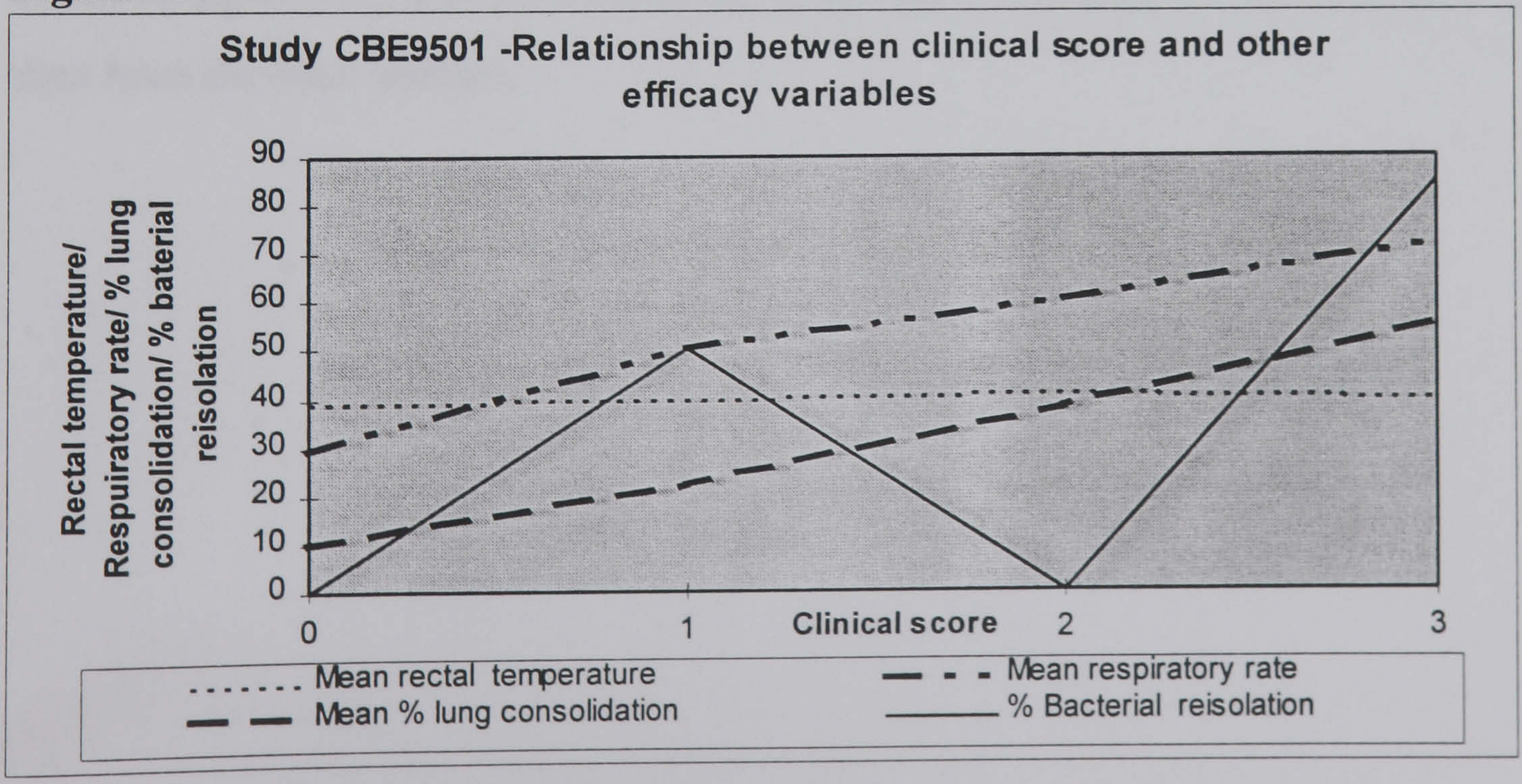
CBE9501						RANKS	RANKS	RANKS	RANKS	RANKS
Clinical score	Calf no.	Rectal Temp.	Resp. score	Lung consolidation (%)	Bacteriol reisolatn	Clinical score	Rectal Temp.	Resp. score	Lung consolidation (%)	Bacteriol. reisolatn
0	5575	39.4	24	0	no	3.5	6.5	1.5	2.5	5
0	9551	39	32	30	no	3.5	4.5	5	9.5	5
0	3597	39	28	0	no	3.5	4.5	3.5	2.5	5
0	5071	39.4	24	0	no	3.5	6.5	1.5	2.5	5
0	7497	39.5	36	30	no	3.5	8	6	9.5	5
0	2382	38.6	28	0	no	3.5	2	3.5	2.5	5
1	7144	38.9	52	15	yes	7.5	3	9.5	6	13
1	6639	40.8	48	30	no	7.5	15	8	9.5	5
2	2772	41	60	90	no	9	16	11	16	5
3	2533	40.2	96	80	yes	13	11	15.5	13.5	13
3	9868	40.4	64	80	yes	13	13	12	13.5	13
3	6720	40.3	84	25	yes	13	12	14	7	13
3	5678	40.6	96	80	yes	13	14	15.5	13.5	13
3	8322	38	52	30	yes	13	1	9.5	9.5	13
3	4627	39.8	40	10	yes	13	9	7	5	13
3	4624	40.1	72	80	no	13	10	13	13.5	5

Discussion (Study 2)

The results described in this study are similar to earlier observations and demonstrate that *P. haemolytica* type A1 is capable of producing severe acute pneumonia in conventionally reared calves (Gibbs *et al.* 1984, Ames *et al.* 1985) after experimental inoculation. The development of clinical signs, gross pathological lesions and bacteriological findings were very similar to those reported in clinical cases of 'shipping fever' (Allan *et al.* 1983, Gibbs *et al.* 1983). With more animals there would have been more likelihood of successful bacterial reisolation at *post mortem* examination of calves scored as "apathetic" (i.e. clinical score 2).

There was an increase in rectal temperature of 0.7 °C between clinical scores 0 and 1 but no further increase in rectal temperature at clinical score 3 (Table 10). However, calves even with clinical score 0 had a higher rectal temperature (mean 39.2°C) than in the previous study (mean 38.3°C) . Although the magnitude of increase in temperature as clinical score increased was less than in the previous study, the calves were more pyrexic (mean temperature of 39.9°C at score 3 compared to 39.8°C in the previous study - the single calf with a clinical score of 2 had a terminal rectal temperature of 41.0°C in comparison with a mean of 39.8°C for calves with a score of 2 in the previous study). The respiratory rate and lung consolidation progressively increased with increasing clinical score (Tables 9 and 10).

Figure 13:



The disease produced by this model appeared to be more severe than in the previous study in terms of the proportion of calves with a high clinical score, mean percentage lung consolidation, and mean rectal temperature. This greater severity may be due to the deep endobronchial infection technique (compared to the trans-tracheal technique used previously). The infection doses were similar to the first experiment, however the simulanteous *Mycoplasma bovis* inoculation might have been expected to act as additional facilitating factor and heighten the disease severity in the previous study. Each of these studies was designed to consider separate scenarios: one, the effect of combined Mycoplasma and Pasteurella infection and the other the effect of transportation stress prior to infection. Experience in repeatedly using the same experimental infection model, however, has shown that even with the same infection technique and doses, there can be a great deal of variation in the results obtained (Reeve-Johnson, 1998a).

Overall, as in the previous study, an increase in clinical score was related to increasing lung consolidation, increasing respiratory rate and bacterial isolation rate. As before, rectal temperature and clinical score, and rectal temperature and respiratory rate were positively correlated. The results in this study were generally supportive of a temperature threshold in the region of 39.7°C as being indicative of the onset of respiratory disease, in accordance with other published recommendations by Bols and de Kruif (1994), Lauridsen *et al.* (1994), and Scott (1994). Formal statistical analysis of the strength of correlation is discussed at the end of this chapter in conjunction with data from the other studies.

Study 3 - (CFR9501) Calves

Overview

Twenty-four Holstein-Friesian or Salers cross calves (6 female and 18 male) with an average weight of 39.8 kg were used in the study. All calves were inoculated on days 4, 5, and 6 with a suspension of *Pasteurella haemolytica* Type A1 by a combination of intra-tracheal and intra-nasal routes designed to simulate the reinfection patterns encountered from 'in contact' animals under commercial housing conditions.

Schedule of study events

Day 0		Arrival of calves, general health inspection, feed electrolyte/glucose solution, weigh all calves
Day 1		Start feeding with milk replacer
Day 4	am	Intra-nasal infection of all calves
	pm	Intra-tracheal infection of all calves
Day 5	am	Intra-nasal infection of all calves
	pm	Intra-tracheal infection of all calves
Day 6	am	Intra-nasal infection of all calves
Day 10		Weigh, euthanase and necropsy calves

Results

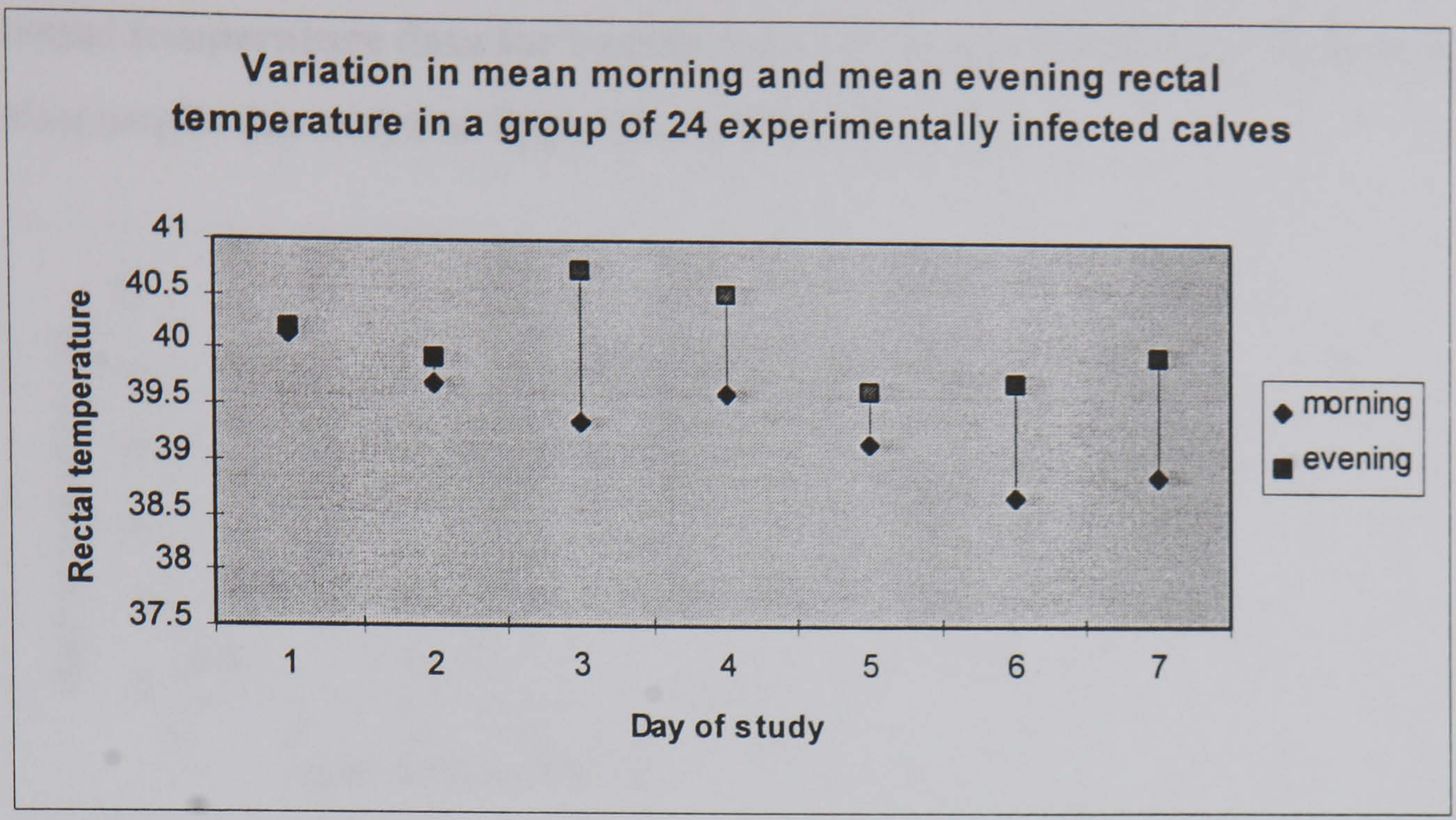
Mortality

Seven calves were euthanased during the course of the study due to severe respiratory disease.

Rectal temperature

At the beginning of the study, prior to the first infection, the mean rectal temperature for the group based on an average of the morning and afternoon readings was 39.9°C (Standard deviation 0.85). After the initial infection, this rose to 40.3°C on the evening after the day of infection (Standard deviation 0.89), however, if the morning temperature is also taken into account, the daily mean was at 39.9°C (Standard deviation 1.0). In 17 of the calves, the temperature declined over a period of 4 days to the initial pre-infection readings. In all seven calves which were euthanased before the end of the study, the temperatures continued to rise. In calf 10, a maximum temperature of 42.2°C was recorded, but this had declined to 39.5°C by the time of necropsy. It was noted that there was regular diurnal variation of the rectal temperature. Rectal temperatures tended to be higher in the evenings than in the morning (Figure 14).

Figure 14:

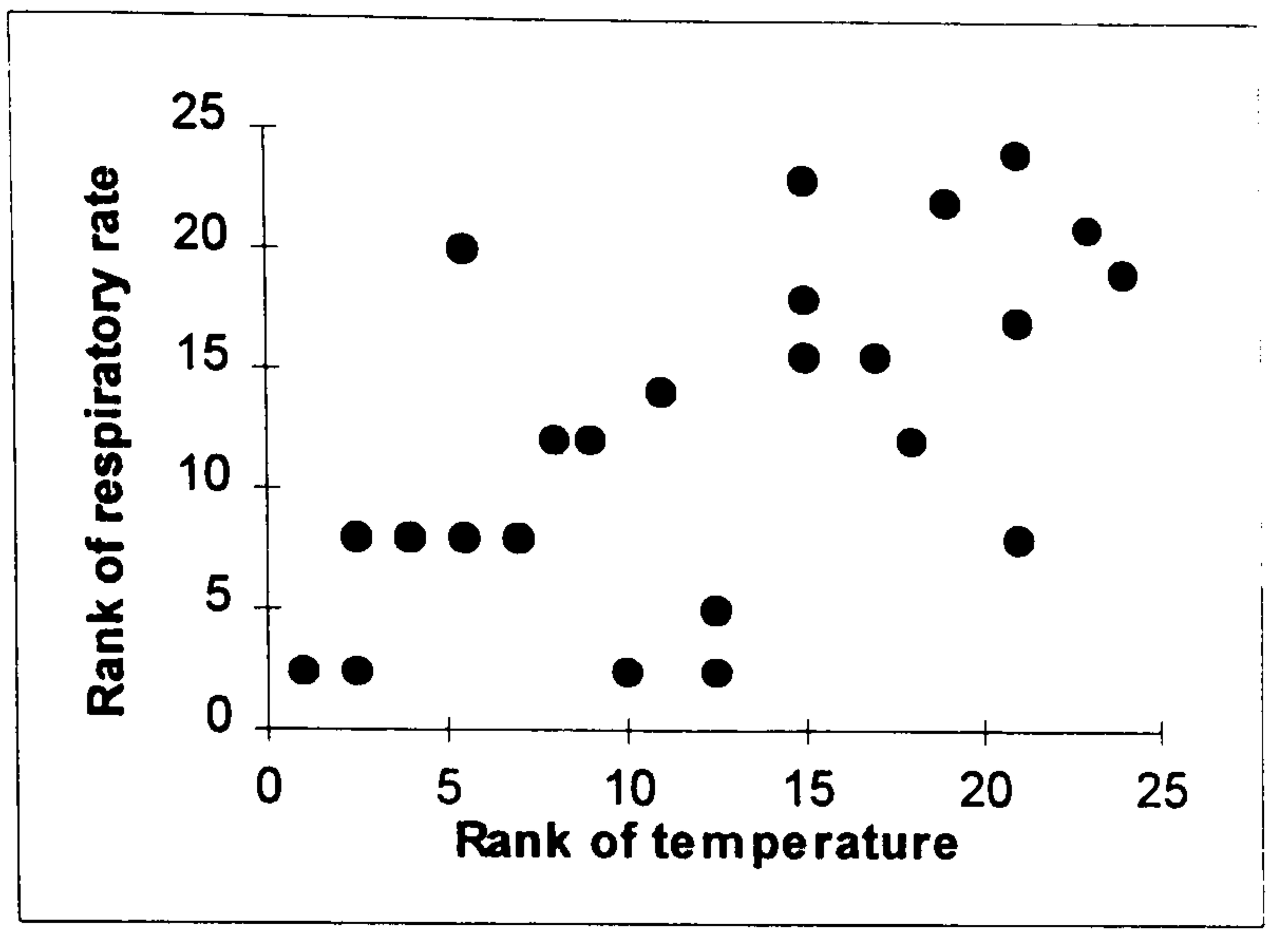


Respiratory rate

A rise in respiratory rate was noted in all calves after the first inoculation day. The pre-infection mean was 28 breaths per minute on the evening of the first day of infection and the day after, the mean was 60 breaths per minute.

The relationship between respiratory rate and temperature is illustrated in Figure 15.

Figure 15: Graphical relationship between ranked respiratory rate and ranked rectal temperature data for twenty-four calves experimentally challenged with *Pasteurella haemolytica* Type A1 on five occassions



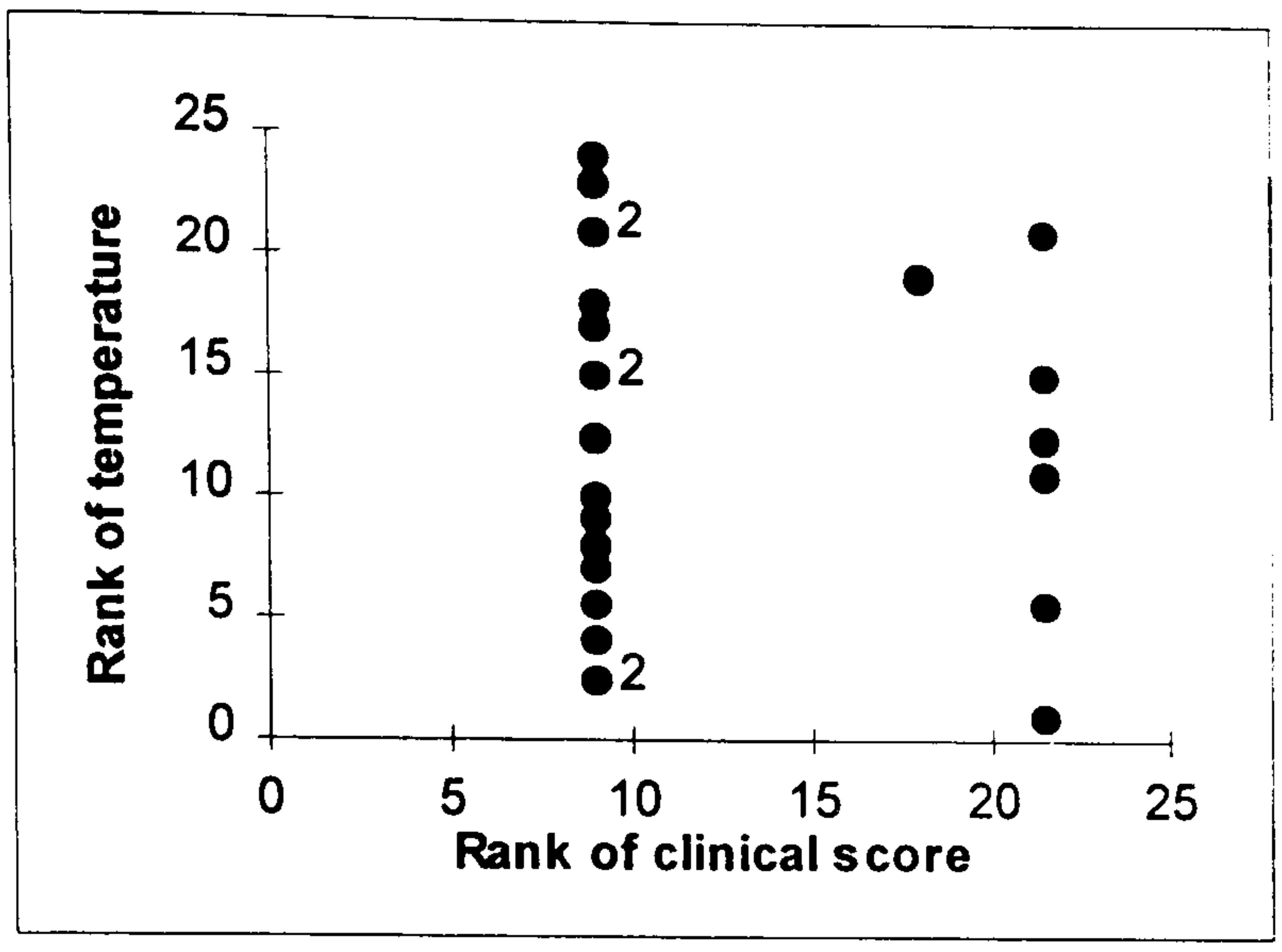
Milk consumption

There was an immediate decline in milk intake after challenge, however intake gradually improved as recovery from infection progressed.

Clinical scores

The clinical demeanour score in all calves was increased immediately after challenge and did not decline in the seven calves which were euthanased; in the other 17 calves it decreased to pre-infection levels by the end of the study. The relationship between rectal temperature and clinical score is illustrated in Figure 16.

Figure 16: Graphical relationship between ranked rectal temperature and ranked clinical score data for twenty-four calves experimentally infected with repeated *Pasteurella haemolytica* Type A1 challenges



N.B. Numbers next to plotted points indicate the number of identical values

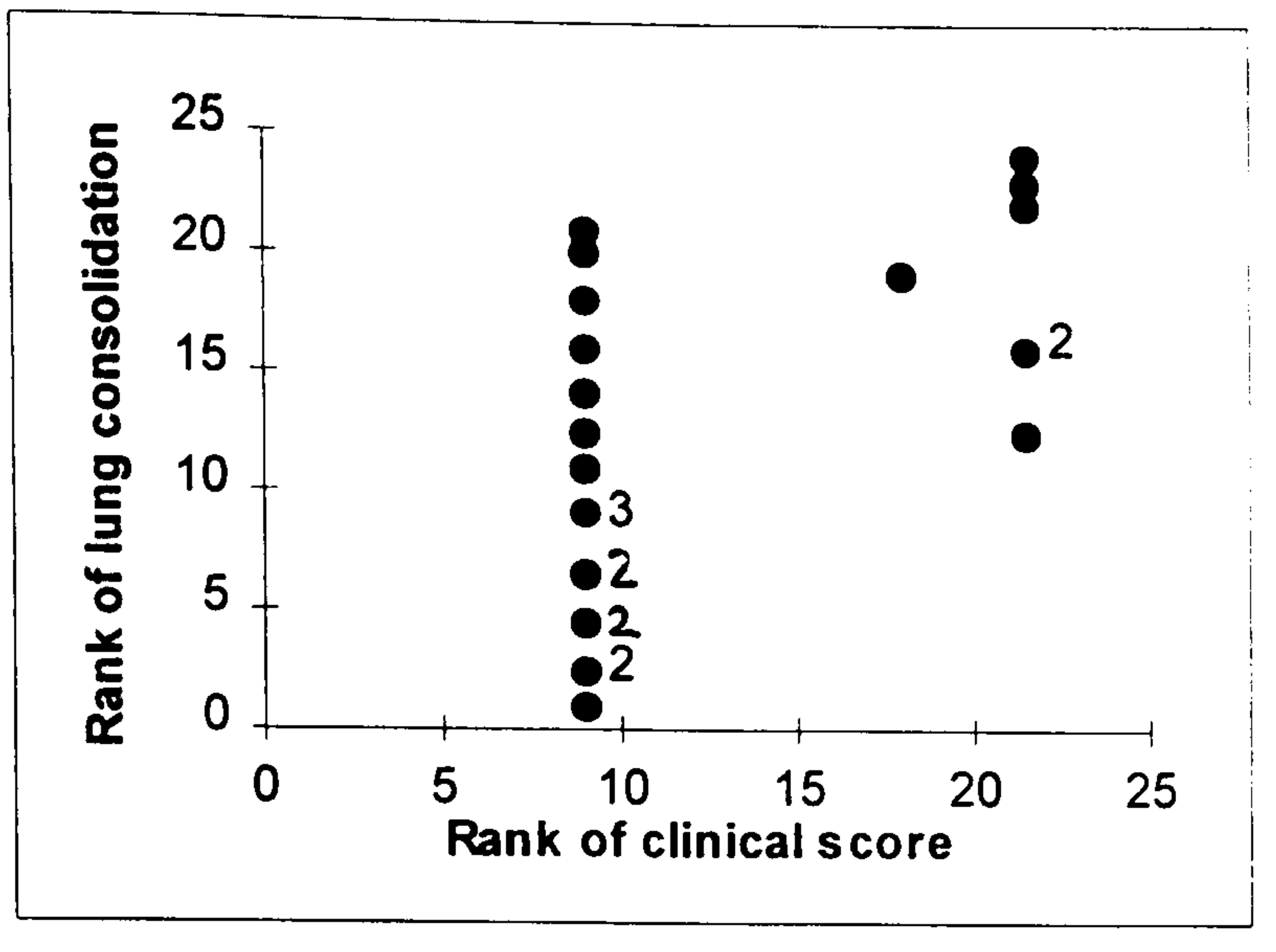
Weight of calves

There were no mean changes in body weight of calves, the trial period was too short for sufficient weight loss to occur and the period of reduced milk intake was short. Calves drinking the least milk were usually those euthanased during the study and therefore they were weighed sooner after experimental challenge than the surviving calves.

Pathology results

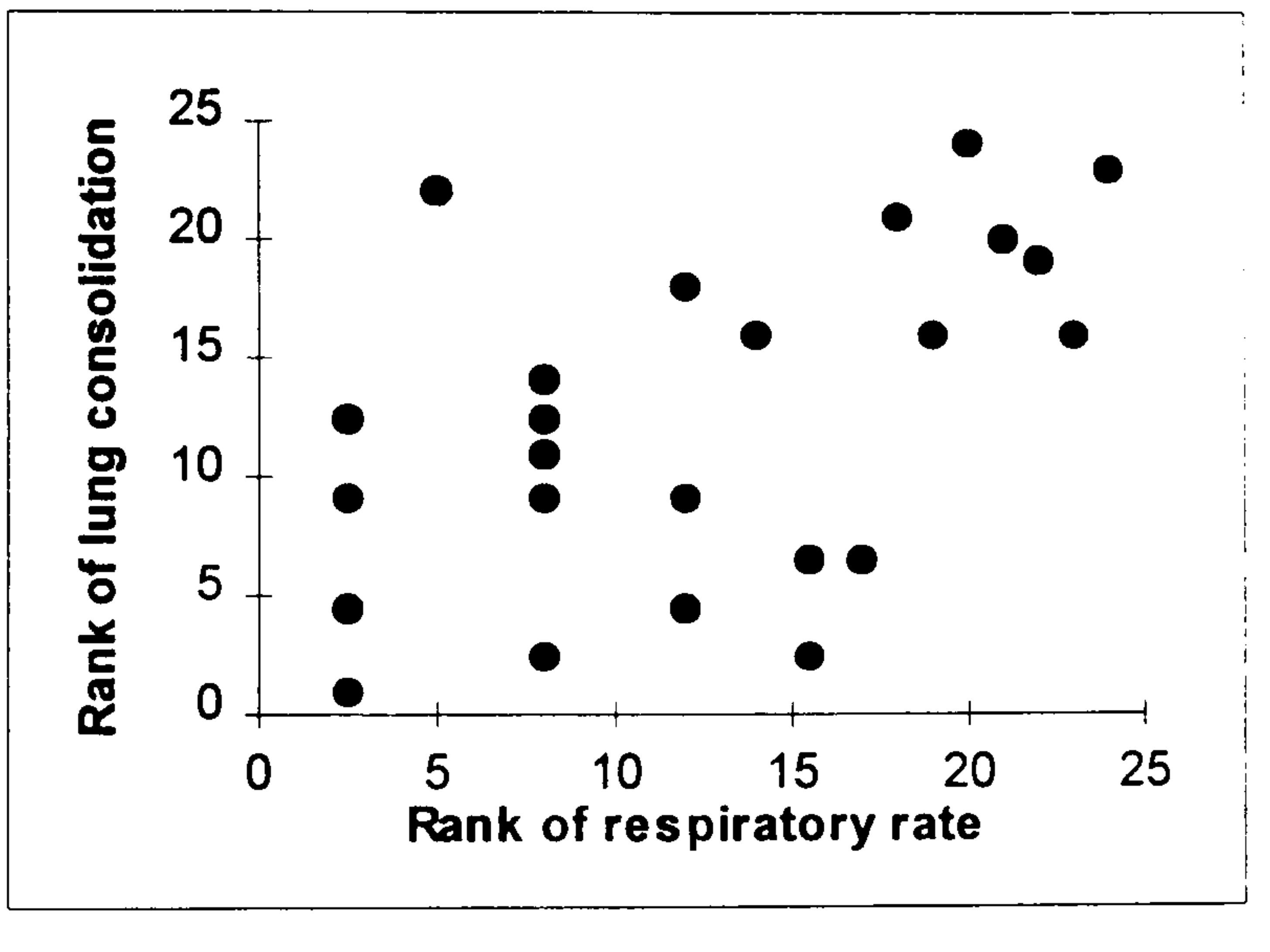
Tables 14 and 15 summarise the lung consolidation observed at necropsy, while Figures 17 and 18 illustrate the relationship between lung consolidation and clinical score, and lung consolidation and respiratory rate respectively.

Figure 17: Graphical relationship between ranked lung consolidation and ranked clinical score data for twenty-four calves experimentally infected with repeated *Pasteurella haemolytica* Type A1 challenges



N.B. Numbers next to plotted points indicate the number of identical values

Figure 18: Graphical relationship between ranked lung consolidation data and ranked respiratory rate for twenty-four calves experimentally infected with repeated *Pasteurella haemolytica* Type A1 challenges



Microbiological results

The number and percentage of calves in each clinical score banding from which *P.haemolytica* or *P.multocida* were isolated from the lung is shown in Table 16. Individual animal results are given in Table 14. A two-way table (Table 14) summarises the relationship between clinical score and bacterial re-isolation.

Table 14: Number of successful reisolations of *P.haemolytica* from calves at each clinical score for twenty-four calves experimentally infected with repeated *Pasteurella haemolytica* Type A1 challenges

Number of animals	Clinical score 1	2	3
Bacterial reisolation			
no	9	0	0
yes	8	1	6

Table 15: Relationship between clinical scores and other variables used to measure severity of disease for twenty-four calves experimentally infected with repeated *Pasteurella haemolytica* Type A1 challenges

T5CCFR Clinical score	Animal number	Terminal rectal temperature	Respiratory rate	% lung consolidation	Bacterial reisolation
1	1	40.1	48	15	yes
1	2	37.5	20	1	yes
1	5	38.6	18	2	no
1	6	39.9	20	9	yes
1	8	37.7	20	6	no
1	9	39.0	34	16	yes
1	10	39.5	24	6	yes
1	13	39.4	30	1	no
1	7	37.5	18	6	no
1	14	39.0	30	5	no
1	15	37.6	20	8	no
1	17	38.9	18	0	no
1	19	38.5	24	13	no
1	20	38.0	24	2	yes
1	22	39.9	31	5	yes
1	23	37.8	20	7	no
1	24	41.2	35	12	yes
2	18	39.8	81	14	yes
3	3	38.8	28	12	yes
3	4	37.1	18	8	yes
3	11	39.9	97	20	yes
3	12	38.9	19	19	yes
3	16	39.0	89	12	yes
3	21	37.7	44	21	yes

Table 16: Summary of relationship between clinical scores and mean rectal temperature, mean respiratory rate, mean percentage lung consolidation and percentage bacterial reisolation for twenty-four calves experimentally infected with five *Pasteurella haemolytica* Type A1 challenges

Clinical score	Number of animals	Mean rectal temperature	Mean respiratory rate	Mean % lung consolidation	% Bacterial Reisolation
1	17	38.8	24	6.3	47
2	1	39.8	81	14	100
3	6	38.5	49	15.3	100

Table 17: Summarised clinical, pathological and ranked data for twenty-four calves experimentally infected repeatedly with intra-nasal and intra-tracheal *Pasteurella haemolytica* A1

CFR9501 Clinical score	Calf no.	Rectal Temp.	Resp. score	Lung consolidn (%)	Bacteriol. reisolation	RANKS Clinical score	RANKS Rectal Temp.	RANKS Resp. score	RANKS Lung consolidn (%)	RANKS Bacteriol. Reisoln.
1	1	40.1	48	15	yes	9	23	21	20	17
1	2	37.5	20	1	yes	9	2.5	8	2.5	17
1	5	38.6	18	2	no	9	10	2.5	4.5	5
1	6	39.9	20	9	yes	9	21	8	14	17
1	8	37.7	20	6	no	9	5.5	8	9	5
1	9	39.0	34	16	yes	9	15	18	21	17
1	10	39.5	24	6	yes	9	18	12	9	17
1	13	39.4	30	1	no	9	17	15.5	2.5	5
1	7	37.5	18	6	no	9	2.5	2.5	9	5
1	14	39.0	30	5	no	9	15	15.5	6.5	5
1	15	37.6	20	8	no	9	4	8	12.5	5
1	17	38.9	18	0	no	9	12.5	2.5	1	5
1	19	38.5	24	13	no	9	9	12	18	5
1	20	38.0	24	2	yes	9	8	12	4.5	17
1	22	39.9	31	5	yes	9	21	17	6.5	17
1	23	37.8	20	7	no	9	7	8	11	5
1	24	41.2	35	12	yes	9	24	19	16	17
2	18	39.8	81	14	yes	18	19	22	19	17
3	3	38.8	28	12	yes	21.5	11	14	16	17
3	4	37.1	18	8	yes	21.5	1	2.5	12.5	17
3	11	39.9	97	20	yes	21.5	21	24	23	17
3	12	38.9	19	19	yes	21.5	12.5	5	22	17
3	16	39	89	12	yes	21.5	15	23	16	17
3	21	37.7	44	21	yes	21.5	5.5	20	24	17

Discussion (Study 3)

The experimental infection model used in this study has been used extensively to determine a variety of pathological phenomena relating to Pasteurella infections (Espinasse *et al.* 1989, 1990a, 1992a, 1993). It has also been used to determine pharmacological parameters in sick calves and to evaluate the efficacy of antibiotic and anti-inflammatory products (Espinasse *et al.* 1990b, 1992b, 1992c). The model has been proven to be reliable in its reproduction with consistent signs and severity of respiratory infection.

The percent lung consolidation increased as the clinical score increased. Due to the single calf with score 2 and no calves with score 0, it is difficult to interpret the gradation in signs. Most data are for calves of score 1 and 3 (Tables 16 and 17). Parameters which increased as clinical score increased from 1 to 3 were: mean respiratory rate, percent lung consolidation and percent bacterial reisolation (see Table 17). The single calf with score 2 had a rectal temperature and respiratory rate above the means for the 6 calves with score 3. This may be an example of a marginal calf being classified as score 2 where it might also have qualified as score 3. This is a reflection of the subjectivity which is always present in any 'clinical impression', even when converted into a score. It could also be that the rectal temperature and respiratory rates may decline as homeostatic control is lost in severely ill calves. However, with data from only a single animal, this could be misleading.

Statistical analysis and discussion of calf study results

Five variables from three studies were available for analysis:

Clinical score (0, 1, 2 or 3)

Rectal temperature (degrees C)

Respiratory rate (breaths per minute)

Lung consolidation (%)

Bacteriological reisolation (0=no, 1=yes)

For the first three of these parameters, the last clinical record prior to *post mortem* examination was used in the correlation analysis. In study CUK9501, respiratory score (0, 1, 2 or 3) was recorded instead of respiratory rate.

The results presented consist of correlation coefficients and p-values for each pair of variables. In each case, Spearman's correlation coefficient was calculated, i.e. given n pairs of observations (x_i , y_i) (i=1,2,...,n) for two variables x and y the coefficient is:

$$\theta = \frac{\sum_{i=1}^{n}(r_i - \bar{r})(s_i - \bar{s})}{\sqrt{\sum_{i=1}^{n}(r_i - \bar{r})^2 \sum_{i=1}^{n}(s_i - \bar{s})^2}}$$

where r_i denotes the rank of x_i, s_i denotes the rank of y_i, and $\bar{r}$ and $\bar{s}$ denote the mean values of the ranks of x and y respectively. The p-value was obtained by calculating the probability of obtaining a correlation at least as big as the one observed for the given number of observations, under the hypothesis of there being no relationship between the two variables. This was done automatically using the CORR procedure in the SAS for Windows (Version 6.12) package.

In each study, there were significant correlations between clinical demeanour score and both lung consolidation and bacteriological reisolation ($p<0.05$). Some of the correlations between other variables revealed greater differences between the studies, e.g. clinical demeanour score with rectal temperature.

On pooling the data, there were significant differences between the three studies in the relationships between the variables. Hence, calculating correlation coefficients for the combined data is not appropriate, and so these results have not been presented.

Spearman's correlation coefficients have been used, as most of the parameters were ordinal but not on a meaningful numeric scale. Hence, non-parametric tests based on the ranks of the data values rather than the actual numbers recorded are more appropriate. Although the use of correlation coefficients with some of the variables is not statistically very meaningful, e.g. correlations involving bacteriological reisolation (recorded as yes or no), the p-values obtained were similar to those from analyses more appropriate to this type of data. For example, correlating a continuous variable such as rectal temperature with bacteriological reisolation could be considered as a two-sample comparison, comparing the average temperature for when bacteriological reisolation is 'yes' and when it is 'no'. Although an appropriate form of analysis, such as a t-test or Wilcoxon Rank-Sum test, would not yield a correlation coefficient, the p-values from the two-sample tests are similar to the p-value obtained from the Spearman's correlation coefficient that has been used. The correlation coefficients involving bacteriological reisolation and other binary responses have been left in for the sake of consistency.

In the situation where there are no tied ranks, the version of Spearman's correlation coefficient used can be simplified to the alternative expression:

$$r = 1 - \frac{6\sum_{i=1}^{n} d_i^2}{n^3 - n}$$

where d_i denotes the difference in ranks for the i^{th} pair of observations. In the situation where tied ranks are present, using this simplified version would cause the correlation coefficents to be slightly biased, and hence the product-moment formula applied to the ranks has been used throughout. The asymptotic distributions used to obtain the p-values are less reliable when there are tied ranks, but in general the results are similar to those that would be obtained using exact methods.

Combined study results

Table 18: Spearman's correlation coefficients for clinical and pathological parameters in calves with experimentally induced pneumonic pasteurellosis

Study	Correlation coefficient (p-value)	Clinical score	Rectal temperature	Respiratory rate	% lung consolidation
1. CUK9501 (n=10)	Rectal temperature	**0.82 (0.004)**	-	-	-
	Respiratory rate	**0.98 (<0.001)**	**0.87 (0.001)**	-	-
	% lung consolidation	**0.86 (0.001)**	**0.69 (0.03)**	**0.87 (0.001)**	-
	Bacteriological reisolation	**0.98 (<0.001)**	**0.87 (0.001)**	**1.00 (<0.001)**	**0.87 (0.001)**
2. CBE9501 (n=16)	Rectal temperature	0.46 (0.07)	-	-	-
	Respiratory rate	**0.86 (<0.001)**	**0.58 (0.02)**	-	-
	% lung consolidation	**0.61 (0.01)**	**0.65 (0.007)**	**0.79 (<0.001)**	-
	Bacteriological reisolation	**0.76 (<0.001)**	0.10 (0.72)	**0.64 (0.007)**	0.24 (0.38)
3. CFR9501 (n=24)	Rectal temperature	-0.06 (0.78)	-	-	-
	Respiratory rate	0.28 (0.18)	**0.62 (0.001)**	-	-
	% lung consolidation	**0.59 (0.002)**	0.25 (0.25)	**0.52 (0.009)**	-
	Bacteriological reisolation	**0.49 (0.01)**	0.37 (0.07)	**0.48 (0.02)**	**0.48 (0.02)**

N.B. Numbers highlighted in bold are significant to $p \leq 0.05$

Numbers in brackets are the confidence interval

In the table of results (Table 18), correlations significant at the 5 percent level have been highlighted in bold. In all three studies, most of the pairwise correlations were significant. Consistent relationships between variables were particularly noticeable in study CUK9501, but the small number of data values in this study should be taken into account. Respiratory rate was correlated with rectal temperature, lung consolidation and bacterial reisolation in each study, but the level of correlation showed some variation between studies. Clinical demeanour score and respiratory rate showed significant correlation in two of the studies (p<0.05), although not in the third. There was only a single animal with a clinical score of 2 which had a very high terminal respiratory rate, above the mean respiratory rates for animals of score 1 or 3. If the data from this single data point are regarded as out-lying, the mean respiratory rate increases from 24 to 49 breaths per minute for the 17 animals with a score of 1 to the six animals with a score of 3 and there would be a statistically significant correlation between clinical demeanour score and respiratory rate.

Clinical demeanour score and lung consolidation correlated consistently, indicating that the pathology evident in the diseased lung directly contributes to the clinical condition of the animals with this respiratory disease.

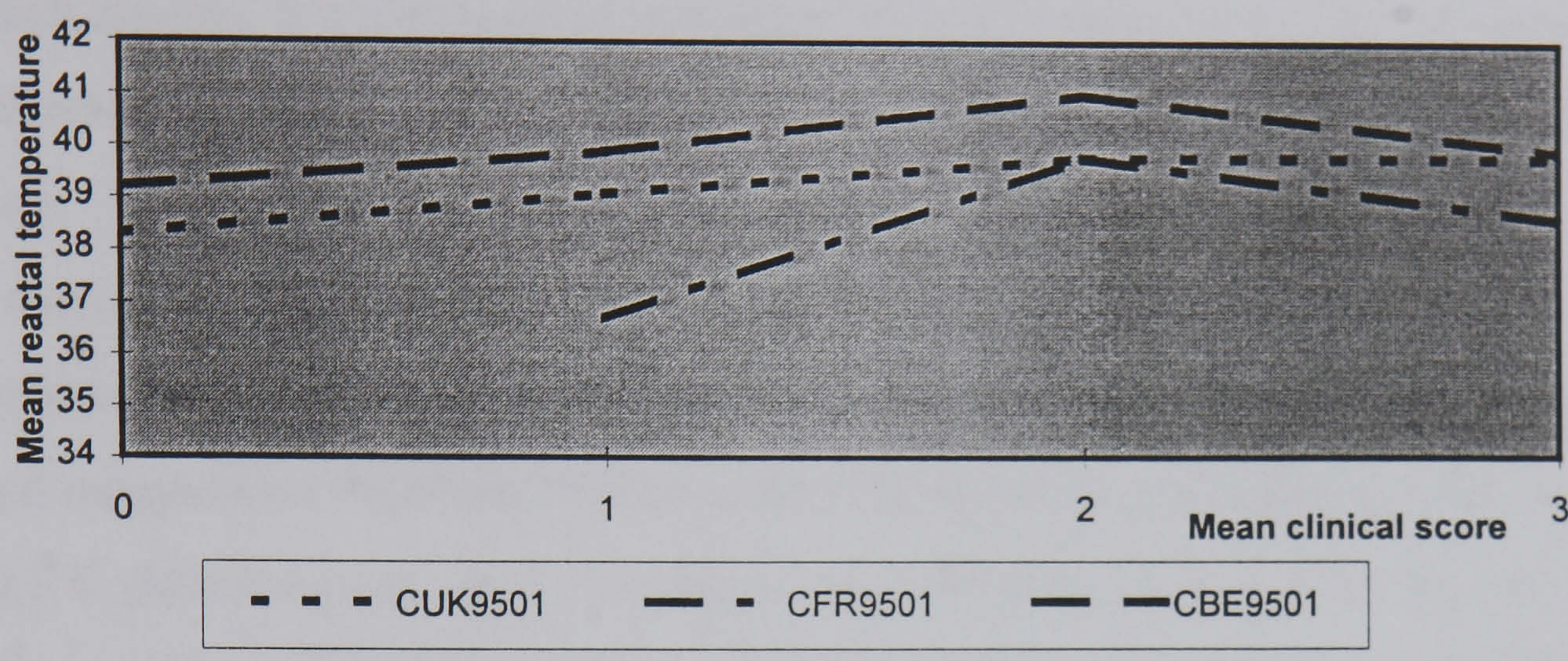
Clinical score and bacteriological reisolation also correlated consistently. It is surprising that there appears to be better correlation with bacterial reisolation and clinical score than clinical score and rectal temperature. This may be due to the release of inflammatory mediators which, although not causing pyrexia, cause deterioration in the clinical condition of animals or, in addition, due to the association of bacterial presence with the quantity of pathological changes in the lung which result in the consequent physiological compromise, reflected by the worsening of clinical condition.

Clinical score and respiratory rate showed consistent correlation. Respiratory rate correlated significantly with both lung consolidation and bacterial reisolation in all three studies. In composite scoring systems, respiratory rate is often considered to be an integral part of the overall clinical demeanour (e.g. Espinasse *et al.* 1989).

There was no direct correlation between clinical demeanour score and rectal temperature in two studies. The explanation for this is best understood in light of the non-linearity seen graphically when clinical scores are plotted against rectal temperature (Figure 20). Although it appears that initially there is good correlation between clinical score and rectal temperature, at high clinical scores the positive correlation ceases, presumably due a loss of homeostatic control of temperature in severely ill animals (score greater than 2). This is a feature in cattle with severe endotoxaemia, e.g. cows with *E.coli* mastitis characteristically may show signs of endotoxic shock and sub-normal rectal temperatures when severely ill (personal experiences).

Figure 20:

Mean rectal temperature plotted against mean clinical demeanour score for each of three experimental infection studies in young calves involving *P.haemolytica*, and in one study (CUK9501) *M.bovis* infection.



N.B. there were no calves with a terminal clinical demeanour score of 0 in Study 3 (CFR9501)

Rectal temperature correlated significantly with respiratory rate, and in two of the three studies with lung consolidation. The lack of significant correlation between rectal temperature in two of the three studies with bacterial reisolation may reflect the importance of the duration that the infection and pathology has been present. Pyrexia is a reflection of the activity of the acute inflammatory response. Bacterial reisolation did not correlate with lung consolidation in one study, possibly for the same reason that, in an inoculation study, large numbers of bacteria are rapidly introduced into the lung, the consolidation, or pyrexic response that might be expected in a field case with a similar bacterial infection load would probably not have had sufficient time to develop with this type of study.

Whilst the relationship between rectal temperature and the progression of other clinical and pathological signs was non-linear, the data could be interpreted that certain threshold rectal temperatures were strongly indicative of disease. This threshold appears to be above 39.5°C, as this was the maximum rectal temperature for any calf with a 'normal' clinical demeanour score of 0. However, there were several calves with higher clinical demeanour scores which had rectal temperatures lower than 39.5°C. The conclusion is that any calf with a rectal temperature above 39.5°C can be defined as 'diseased', but a rectal temperature lower than 39.5°C does not mean that the calf is not diseased. These findings support those of Scott (1994, 1996, 1998), with the caveat that calves with a rectal temperature below 39.5°C should not be considered out of risk. This threshold is still of practical use, however, as these studies showed that calves with rectal temperatures below 39.5°C, but diseased as evidenced by pathological and clinical evidence, had positive clinical demeanour scores. The combination of rectal temperature monitoring and observation of animals for clinical signs should therefore be a practical method of detecting animals with respiratory infections associated with *P.haemolytica*. The data indicate that the high rectal temperature thresholds which some workers have recommended, for example >40.5°C (Mechor *et al.* 1988, Gorham *et al.* 1990, Picavet *et al.* 1991, Morck *et al.* 1993) would not detect the majority of diseased calves. Across the three studies, out of 16 calves with 'severe disease' (i.e. a clinical demeanour score of 3) only one had a rectal temperature above 40.5°C. Only one of the three calves with an 'apathy' clinical demeanour score 2 had a rectal temperature above 40.5°C. Two out of the 19 calves with a 'subdued' clinical demeanour score of 1, had a rectal temperature above 40.5°C.

The three studies differed in infection technique, dose and route of infection, and in one case a combination of infecting organisms was used. Consistent trends in the relationships between clinical and pathological data in individual studies, despite different infection methodologies, suggests that under different clinical disease scenarios, similar relationships might be expected.

Chapter 3: Experimental infection of chickens and the evaluation of disease severity

Introduction

In poultry, respiratory disease due to mycoplasma infection is a significant cause of production loss in the absence of treatment (Le Turdu *et al.* 1984, Dudouyt 1987). Mycoplasmas, which belong to the class Mollicutes, are the smallest self-replicating procaryotes. Twenty-four species of the genus Mycoplasma have been recovered from avian sources, but only four have been established as pathogens for domestic poultry, namely, *Mycoplasma gallisepticum* and *Mycoplasma synoviae* for chickens and turkeys, *Mycoplasma meleagridis* for turkeys and *Mycoplasma iowae* for turkey embryos. The pathogenicity of other species has not been demonstrated except under experimental conditions (Jordan 1990). In many parts of the world, *Mycoplasma gallisepticum* infection remains a primary cause of respiratory disease and is responsible for chronic respiratory disease (CRD) in chickens. Infection is transmitted by direct contact or via the air to birds in close proximity. There are variations in clinical signs but these often include "snicking"[1], in some cases swollen infra-orbital sinuses from which exudate can be expressed, and increased flock mortality. Clinical outbreaks may be precipitated by a number of viral causes (Boden 1993). Secondary infection with *Escherichia coli* is frequent, and is largely responsible for the perihepatitis and pericarditis often seen in mycoplasma-infected birds. Mycoplasma are fragile organisms and do not survive for protracted periods in a hostile environment (Bradbury *et al.* 1996), which causes inevitable problems in their transport for culture and reisolation.

Typical gross pathology involves the infra-orbital sinuses and changes to the airsacs. The airsacs are transparent in the healthy subject but with Mycoplasma infection, they become progressively opaque, neo-vascularised, the walls thicken with inflammation,

[1] This characteristic clinical sign is a wide gaping of the beak, accompanied by a coughing motion with a clicking sound

nodules of purulent material may form in them and the walls may become lined with purulent material (Figures 1 to 5).

Typical pathological signs may be masked by supra-infection with secondary organisms such as environmental *E. coli* or *Aspergillus fumigatus* (Boden 1993). Maintenance of birds in tightly controlled environments, and infecting them with pure *Mycoplasma gallisepticum* is the most practical way of assessing the primary pathology and clinical signs due to infection with this organism.

The disease is considered to be an important problem for broilers and economic losses are significant: up to 20-30 percent reduction of weight gain, 10-20 percent decrease of feed conversion efficiency, 5-10 percent mortality and 10-20 percent condemnation of carcasses at processing (Kempf, I., 1998 -personal communication). In 1986, poultry meat accounted for 23.6 percent of all meat consumed in the world, 19.4 percent of meat consumed in Europe and 37.8 percent of meat consumed in North America (Anon 1993). Any disease impairing production therefore has the potential to cause massive economic losses.

Materials and Methods

The study was conducted using experimentally-induced *Mycoplasma gallisepticum* infection in specific-pathogen-free chickens, using 36 replicated pens of 10 birds. At 6 days of age, ten birds were allocated to each of 36 pens. Birds were distributed so as to attain an approximately equal mean weight in each pen and identified by means of wing bands. Birds were inoculated at ten days of age and were observed for 11 days post-challenge. Necropsy was performed on each bird during which assessment of lesions and sampling for mycoplasma culture and serology were performed. Bodyweight gains of the different groups were compared.

Experimental birds

Species	Chicken
Breed	White Leghorn
Number	360
Sex	180 male and 180 female
Weight on Day 1	34 to 45 g
Health Status	Specific-pathogen-free chickens (see Appendix for list of pathogens)
Inclusion/exclusion criteria	Only healthy chicks were included in the study

The chicks were housed in a building which was purpose-built for experimental work involving the containment of pathogens and maintenance of Specific-Pathogen-Free (SPF) status. Pressure controlled animal rooms with filtered air and controlled temperature were used. The rooms were maintained to provide a temperature of approximately 30°C at chick level initially, dropping to approximately 25^{0}C after approximately two weeks. Temperature and schedules of light were monitored daily throughout the study. The feed and water were checked and recorded each morning and evening, when the chicks were examined for clinical signs. See Appendix for a description of the disease containment facilities in the Animal Experimental House.

A chick-feed free of antibiotics, coccidiostats and other chemical or biological growth promoters was prepared. Chicks were fed *ad libitum* in feed troughs allowing adequate trough space per bird.

Changes in water intake are sometimes used by field clinicians as an indicator of the beginning of a respiratory disease outbreak in commercial flocks. During the trial water was provided *ad libitum* in one bell drinker per cage. Each replicate had its own drinker. The water remaining after 12 hours was measured in a 1 litre measuring cylinder to the nearest 10 ml and the quantity consumed daily by each group was calculated for the period from two days before infection until three days after infection. Remaining water was discarded and fresh water was used to fill the drinkers.

Challenge organism

Organism	*Mycoplasma gallisepticum*
Strain	R-PLO
Source	Chicken trachea from field outbreak
Pass No	2 passages after reisolation from chicken trachea
Storage details	-70°C ± 3°C
Culture details	According to the Standard Operating Procedure "Growth of *Mycoplasma gallisepticum* R-PLO" (See Appendix)
Transport to unit	In double sealed containers in ice.
Dose level	Approximately 10^7 cfu total dose in a volume of 0.25 ml
Administration	0.2 ml per bird intratracheally and 0.05 ml per bird into the sinus
Post-challenge analysis	An aliquot of challenge material was returned to the laboratory for dose reconciliation

Non-infected birds were similarly inoculated with sterile mycoplasma broth. Major study activities are summarised in the schedule of events below.

Schedule of Events

Age (days)	Event
0	Hatching: sampling of 20 sera from spare chickens for mycoplasma serology
6	Identification of all chicks with wing-tags Randomisation of groups and replicates according to weight stratification. Allocation of birds to pen replicates
8	Measurement of water consumption for each replicate.
9	Measurement of water consumption for each replicate
10	Intratracheal and intrasinus challenge with *Mycoplasma gallisepticum* Intratracheal and intrasinus challenge with sterile broth of control birds
11	Measurement of water consumption for each replicate
12	Measurement of water consumption for each replicate
13	Individual weighing of chicks Individual observation of respiratory signs and measurement of water consumption for each replicate
16	Individual observation of respiratory signs
20	Individual weighing of chicks Individual observation of respiratory signs Euthanasia of non-infected birds, observation of lesions, sampling for mycoplasma recovery and serology
21	Euthanasia of infected birds, observation of lesions, sampling for mycoplasma recovery and serology

Observations and Monitoring

On the first day of life, serum was collected from 20 spare chicks *for Mycoplasma gallisepticum* antibody analysis. Birds were inspected and weighed at six days of age. During the study the mortality per group was recorded daily. Non-infected birds and infected surviving birds were killed on days 20 and 21, respectively. Birds were examined for lesions of the peritoneum and air sacs and trachea samples were taken for mycoplasma recovery. Air sac, peritoneal, pericardial and peri-hepatic lesions were scored and recorded.

Clinical Examinations

No clinical demeanour scoring techniques were found in the published literature for live chickens. Despite attempts to develop such a scoring system for clinical signs, with the exception of respiratory signs, it was not possible to identify suitable clinical variables for use in assessing clinical demeanour. A 'respiratory score' was therefore developed. The respiratory signs were recorded and quantified for individual chickens as follows (Kempf *et al.* 1997, Reeve-Johnson *et al.* 1997a):

0	**no respiratory signs**	no rales upon auscultation with ear firmly applied to thorax of bird, no clinical signs, before or during handling.
1	**slight signs**	mild rales upon auscultation with ear firmly applied to thorax of bird, absence of or only intermittent signs of snicking or dyspnoea only evident upon handling the bird.
2	**moderate signs**	clear rales upon auscultation may also be detected with ear withdrawn a short distance from bird, dyspnoea, mild depression or snicking evident before handling bird, worsened by handling.
3	**severe signs**	Clear rales upon auscultation, audible with ear removed from thorax of bird, clinical signs clearly evident in the bird before handling, profound and constant dyspnoea, depression, possibly accompanied by recumbency and 'snicking'.

Pathological scoring system

The airsacs of birds were scored at necropsy using a system based on that first described by Kleven *et al.* (1972), and adapted by Kempf *et al.* (1997) and Reeve-Johnson *et al.* (1997a).

Lesions of air sacs were scored as follows:

0	no visible lesions
1	cloudiness of air sacs
2	air sac membranes thickened and opaque
3	consolidated appearance of membranes with large accumulations of 'caseous' exudate confined to one airsac
4	as 3 but lesions in 2 or more air sacs

Lesions of the peritoneum were scored as follows based on the system described by Reeve-Johnson *et al.* (1997a):

0	no visible lesions
1	cloudiness of peritoneum
2	peritoneum thickened and opaque
3	consolidated appearance of membranes with limited accumulations of caseous exudate
4	as 3 but with large accumulations of 'caseous' exudate

Photographs of representative lesions caused by *M.gallisepticum* were taken from birds which had been infected with *M.gallisepticum* as part of the preparation for this study (Figures 1-5). These were used as an aid to ensure consistency during necropsy when scoring lesions. Due to its ubiquitous presence in the litter and in the environment in general, *E.coli* is a major secondary pathogen in complicated respiratory disease [CRD] in chickens. To help distinguish pathological changes due to *M. gallisepticum* from those due to *E.coli* a picture showing pathognomonic lesions for this organism, which invariably involves signs of inflammatory changes around the liver, was also taken from a previously experimentally infected bird for use a guide to recognising these (Figure 6). The typical fibrous 'tagging' on the liver surface is termed perihepatitis.

Perihepatitis score:

0 No evidence of inflammatory changes seen on liver surface
1 Small discrete areas of inflammatory changes on <50% of liver surface
2 Extensive areas of fibrous 'tagging' and inflammatory changes on the surface of the liver covering >50% of the liver surface

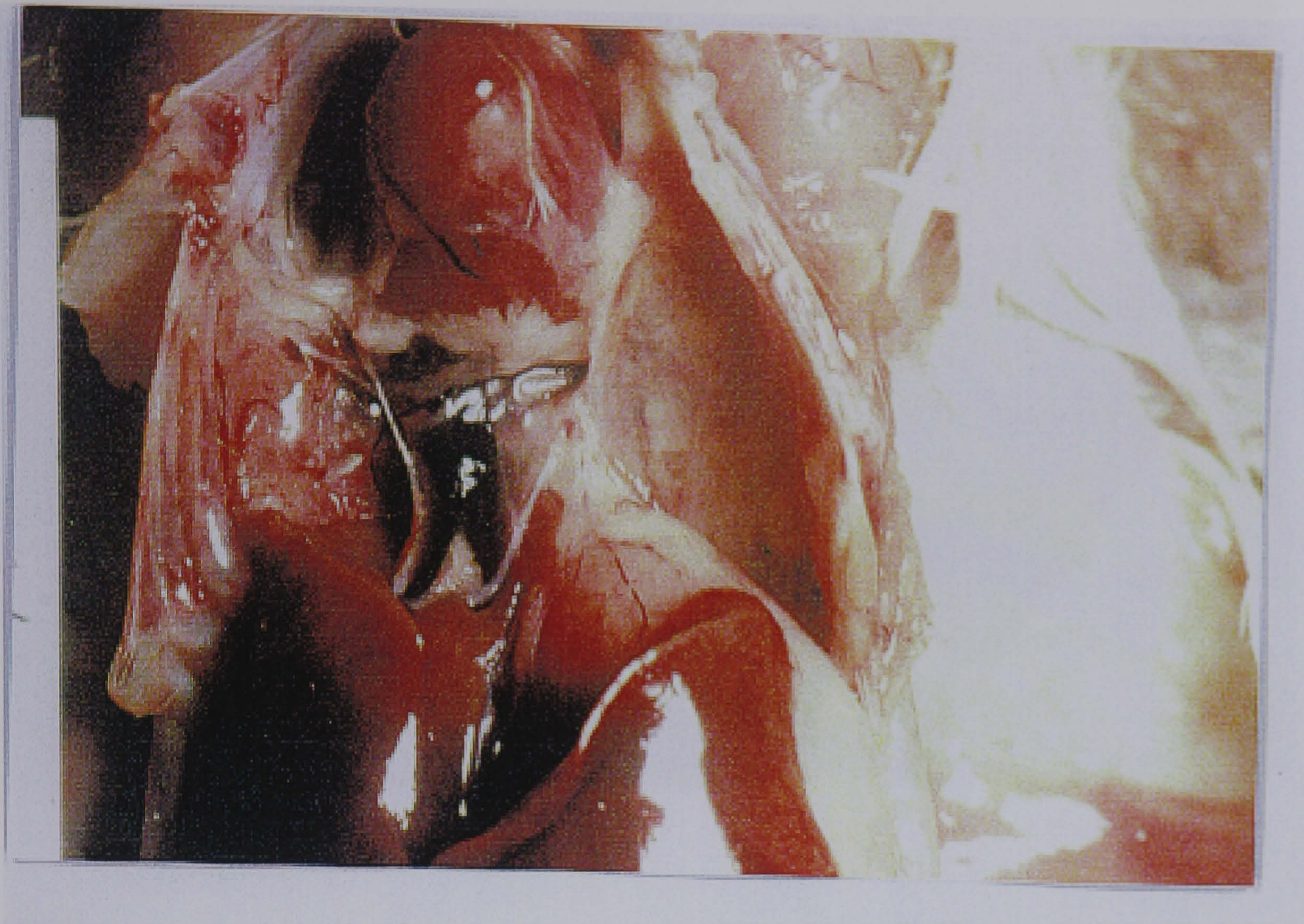
Pericarditis score:

0 No evidence of inflammatory changes observed on the pericardial surface
1 Small discrete areas of inflammatory changes present on <50% of pericardial surface
2 Extensive evidence of inflammatory changes on >50% of the pericardial surface

Figure 1: Illustrating airsac lesion score 1 and peritonitis lesion score 0:

<u>Note</u>:
1 Cloudiness of airsac
2 No exudate present
3 No perihepatitis or pericarditis

Figure 2: Illustrating airsac lesion score 2 and peritoneum lesion score 0:

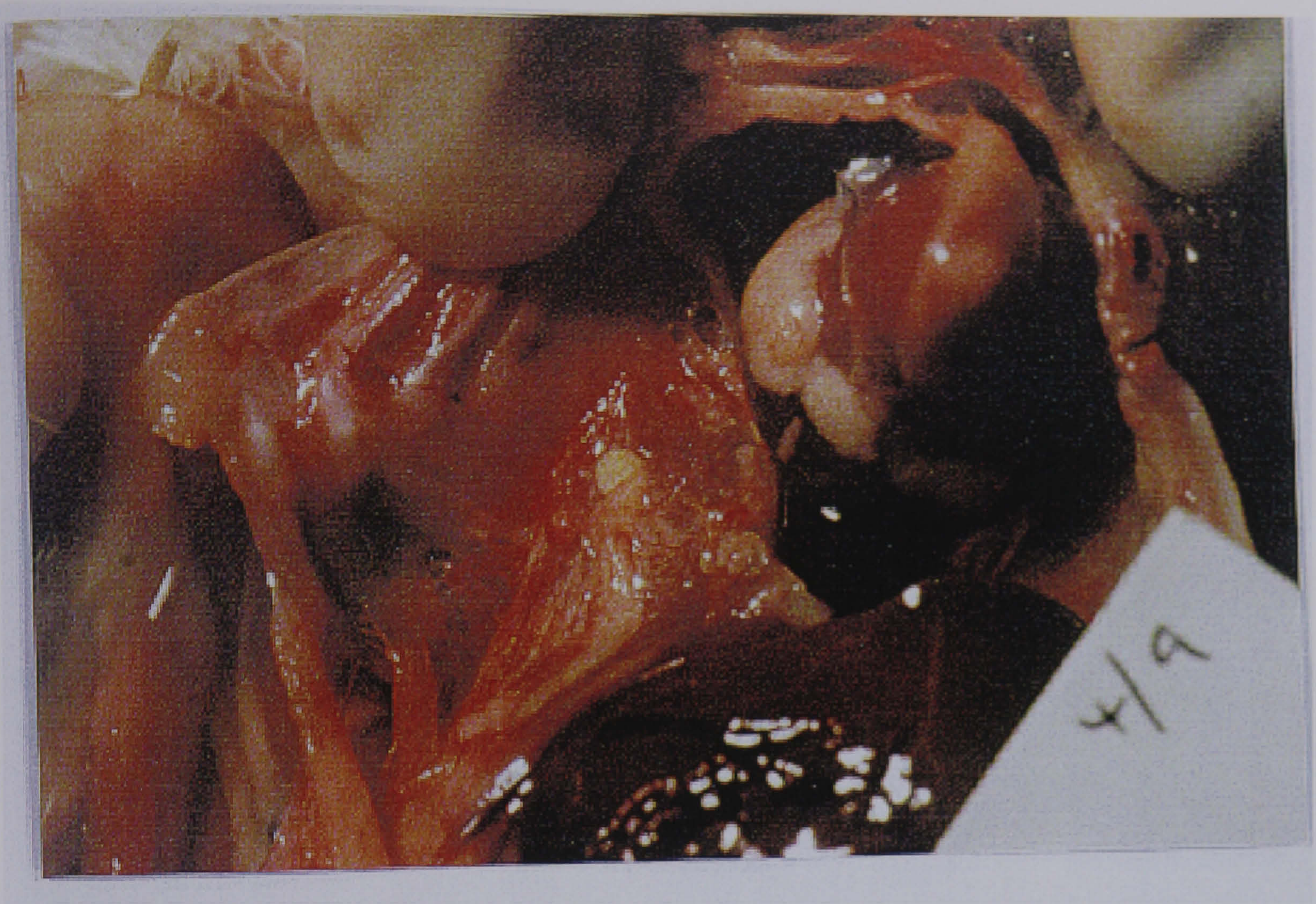


Note: Right airsac score 0, clear, with some neo- vascularisation, but otherwise clear

Left airsac score 2, thickened airsac, but no exudate present

There are no signs of perihepatitis or pericarditis

Figure 3: Illustrating airsac lesion score 2-3 and peritoneum lesion score 0:

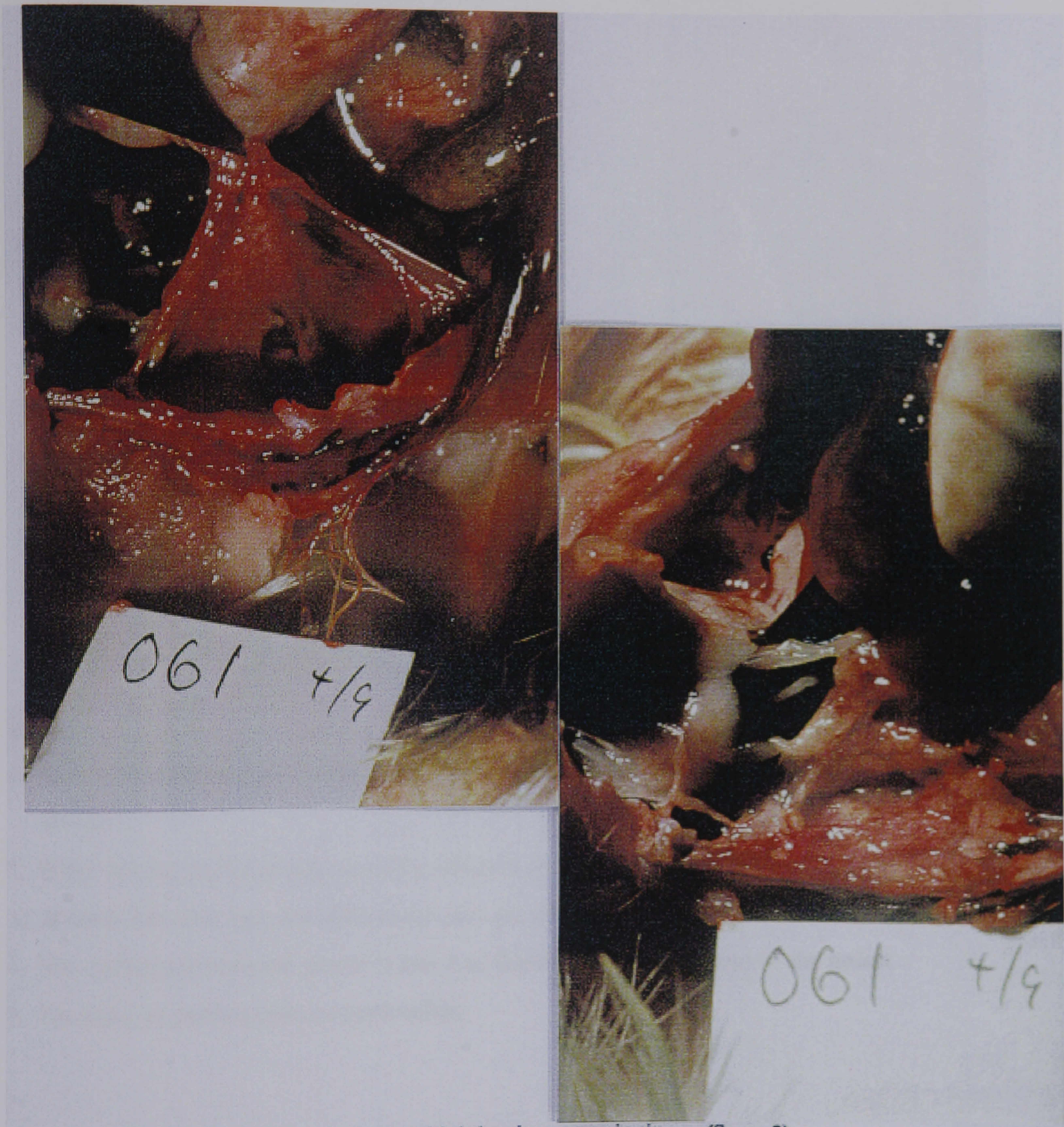


Note:

1. This was scored as 2 because the airsac is thickened but not consolidated
2. Small amounts of exudate are present
3. No signs of perihepatitis or pericarditis

Figure 4: Illustrating lesion airsac lesion score 3 and peritoneum lesion score 0:

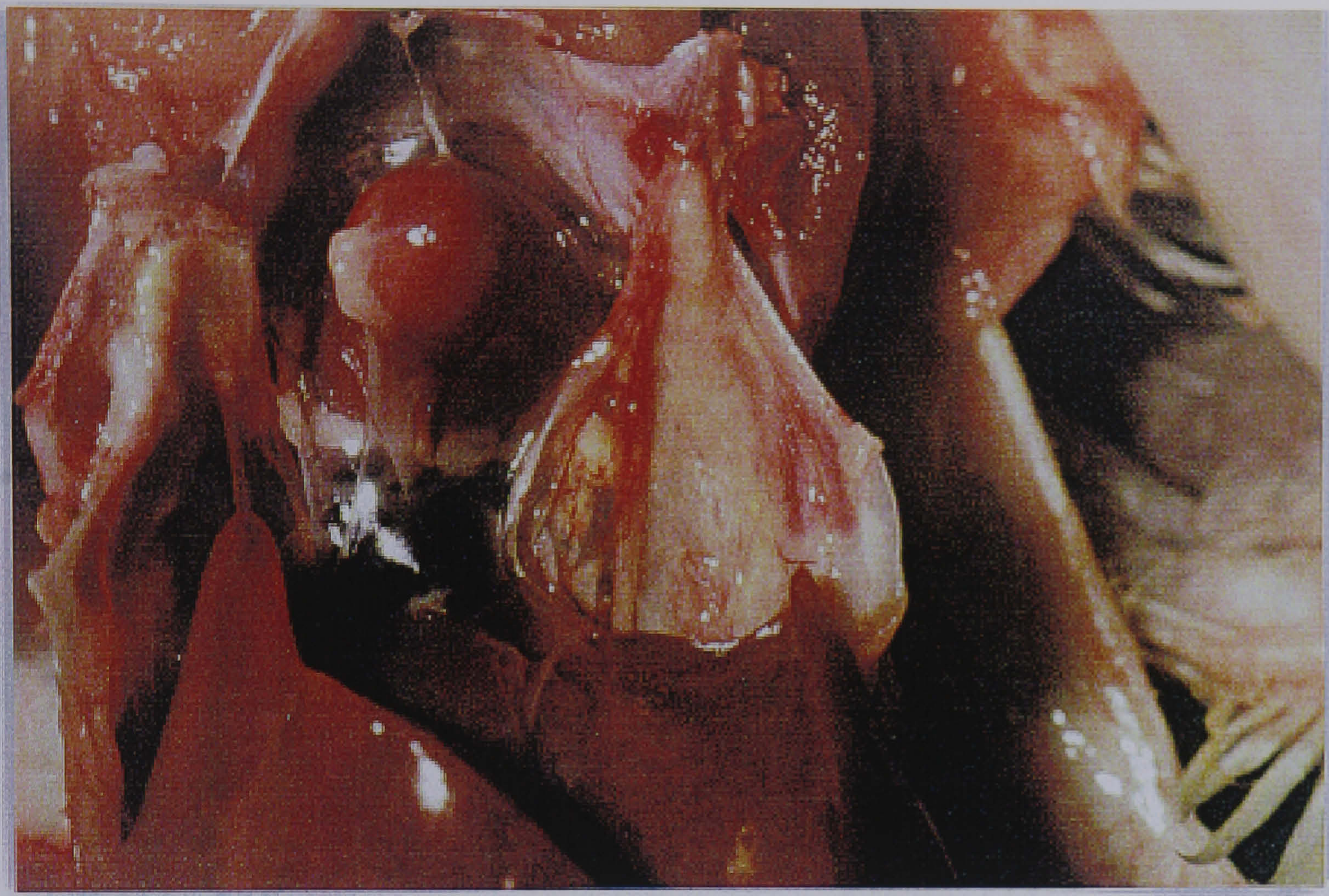
Air sac extended to show thickening and consolidation



Same bird showing organs in situ. **(Score 3)**

Note: airsac thickening and exudate present, but peritoneum, pericardium and liver surface show no signs of gross pathological changes

Figure 5: Illustrating airsac lesion score 4 and peritoneum score 0:



Note:

1. Left air sac (extended) showing typical thick 'meaty' appearance with exudate in air sac
2. Right air sac *in situ* is also severely affected with similar changes
3. In birds like this one, the abdominal sacs are often also affected
4. The difference between scores 3 and 4 is determined by the extent of the lesion
5. No signs of perihepatitis or pericarditis

E.coli infected birds:

Figure 6: Illustrating typical changes seen with secondary bacterial invasion by *E.coli:*

Note:

1. Airsac score 4
2. Pericarditis score 2
3. Perihepatitis score 2
4. Lesions such as this, often with accompanying pericarditis are evidence of bacterial invasion

Mycoplasma recovery

Examination of *post mortem* cultures for *M.gallisepticum* was performed according to the SOP "Recovery of *Mycoplasma gallisepticum* R-P1O" (see Appendix).

Mycoplasma serology

Sera were tested by the slide agglutination test according to SOP "Serological slide agglutination tests for *Mycoplasma gallisepticum*" (see Appendix)

Results

The severity of clinical signs and pathological lesions was clustered according to replicate. This was because of the close proximity of birds and environmental uniformity for all individuals in the same replicate. Under commercial conditions, prevalence of clinical signs is considered on a group basis.

According to the severity of clinical signs on day 13, pens were ranked according to the severity of respiratory signs as indicated by the mean score for each pen. For statistical interpretation, the pens were then banded in groups of six, thus giving five degrees of freedom for the analysis of correlation data.

Titre of the inoculum

The titre of the inoculum was verified as 10^8 CCU/ml

Mortalities

The total number of deaths was 5 out of 360 birds (1.4 percent).

Two birds (No 366 and 135) died on days 20 and 21 (groups 1 & 2). *Post mortem* examination revealed severe lesions of the air sacs, peritoneum and trachea in both birds. One bird died on day 16 (No 341) and two other birds from this group (6) died on day 20 (No 313 and 193). Only a slight lesion of pneumonia was seen in one of the chickens that died on day 20.

Clinical examinations

No clinical signs were observed in non-infected birds.

The first respiratory signs were observed on Day 13: a few chickens showed dyspnoea and sneezing. On the following days, and until the end of the experiment, these signs remained frequent.

The mean respiratory scores for days 13, 16 and 20 of the study (i.e. 3, 6 and 10 days after infection) are given in Table 1.

Table 1: Mean group respiratory scores (with Standard errors in brackets) for six groups each of 60 chickens at 3, 6 and 10 day intervals after experimental infection with *M.gallisepticum*.

GROUP	Day 13	Day 16	Day 20
1 non-infected	0.00 (0)	0.00 (0)	0.00 (0)
2	0.53 (0.12)	1.05 (0.11)	1.85 (0.14)
3	0.38 (0.11)	0.47 (0.19)	0.30 (0.12)
4	0.30 (0.13)	0.47 (0.16)	0.33 (0.11)
5	0.23 (0.09)	0.50 (0.06)	0.38 (0.19)
6	0.15 (0.14)	0.65 (0.17)	0.47 (0.15)

Note: Groups were comprised of six replicated pens of 10 chickens. The six uninfected pens of control birds were assigned to group 1. The remaining replicated pens were ranked according to the severity of respiratory signs on day 13. Six pens were then allocated to each group in order of decreasing severity of signs. Thus the six pens with most evidence of respiratory disease to group 2, the next six to group 3, until the last six pens contained the inoculated birds showing least evidence of respiratory disease and these were allocated to group 6.

Water consumption (see Table 2)

There was a consistent decrease in water consumption after infection in all groups. In comparing the mean water consumption data for day 10 (day of inoculation) with day 11, the decline in consumption ranged from 1.3 percent (Group 3) to 27 percent (Group 4). There was a decline in water consumption of 19 percent in the sham-inoculated birds between days 10 and 11, thus it was not possible to distinguish between the severity of clinical signs based upon water intake alone. Handling the birds and the sham-inoculation procedure appeared to affect the water consumption of birds as much as the presence of an infecting organism. When the mean group water consumption for day 9 (prior to inoculation) and the means of days 11, 12 and 13 (the three days immediately following inoculation) were compared, there was still a notable decrease in water intake. The lowest decrease in water intake was in the control (sham-inoculated) birds i.e. 6 percent (Group 1), whilst the depression in water intake in groups 2-6 were 10 percent, 12 percent, 19 percent, 9 percent and 7 percent respectively.

Table 2: Daily water consumption (milli-litres) for six groups of 60 birds in descending order of disease severity as measured by severity of respiratory signs on day 13 (infection with *M.gallisepticum* occured on day 10 of the study)

GROUP	PEN	Day 9	Day 10	Day 11	Day 12	Day 13
1 (Non-infected)	a	730	520	470	485	330
	b	700	770	370	650	500
	c	400	410	400	530	320
	d	390	500	460	470	330
	e	440	430	500	600	360
	f	450	640	530	1080	410
	mean	**518**	**545**	**455**	**636**	**375**
	Std. Error	141	125	55	208	63
2	a	530	360	340	370	400
	b	450	400	420	460	490
	c	240	280	440	430	360
	d	520	330	300	380	410
	e	440	350	240	310	460
	f	390	440	360	370	450
	mean	**428**	**360**	**350**	**387**	**428**
	Std. Error	97	51	68	121	43
3	a	530	510	430	460	430
	b	570	510	350	460	500
	c	410	390	310	550	590
	d	400	380	260	320	570
	e	420	350	270	400	400
	f	390	380	270	330	400
	mean	**453**	**420**	**315**	**420**	**482**
	Std. Error	70	65	60	80	77
4	a	690	420	360	520	630
	b	460	390	300	330	380
	c	400	400	320	500	530
	d	440	350	280	410	430
	e	410	440	350	330	360
	f	380	370	250	340	360
	mean	**463**	**395**	**310**	**405**	**448**
	Std. Error	105	30	38	79	100
5	a	490	400	460	470	460
	b	420	380	300	410	420
	c	330	330	270	350	370
	d	470	400	340	440	520
	e	390	440	310	340	340
	f	390	360	320	400	310
	mean	**415**	**385**	**333**	**402**	**403**
	Std. Error	53	35	60	46	72

6	a	430	400	340	410	400
	b	390	290	260	350	360
	c	300	310	240	330	340
	d	390	350	270	380	360
	e	420	400	270	430	420
	f	370	330	310	480	510
	mean	**383**	**347**	**282**	**397**	**398**
	Std. Error	42	42	33	50	57

Bodyweight changes (See Tables 3 and 4)

The heaviest bird weights on day 20 were recorded in the control group (sham-inoculated/ uninfected birds). The birds with the most severe signs (group 2), despite having had the heaviest mean weight on day 13, were the lightest by day 20 (Table 3). There was little difference in bodyweights between groups 3-6.

The birds in group 2 initially also had a higher rate of bodyweight gain between days 6-13, attributed to a random affect during allocation. The bodyweight gain for the post-infection phase of group 2 (days 13-20) is markedly lower than any of the other groups and they also have the lowest gain over the whole trial period (Table 4). There was little difference in bodyweight gain for birds with milder signs (groups 3-6), however all had a lower gain than the control group of birds (Table 3).

Table 3: Mean bodyweights (grams) of six groups of 60 birds, four days before experimental inoculation with *M.gallisepticum* and three and ten days after inoculation (standard error in brackets)

GROUP	Day 6	Day 13	Day 20
1 (non-infected)	56.20 (0.2)	115.90 (2.4)	208.30 (4.5)
2	56.15 (0.1)	120.90 (3.0)	187.05 (4.8)
3	56.10 (0.1)	113.25 (1.4)	199.00 (3.9)
4	56.15 (0.4)	114.00 (2.2)	202.40 (5.6)
5	55.90 (0.1)	113.50 (1.1)	199.80 (3.7)
6	56.10 (0.2)	112.00 (1.3)	199.40 (5.4)

N.B. Each group consisted of six replicated pens of 10 chickens

Table 4: Mean body weight gains (grams) of six groups of 60 chickens experimentally inoculated with *M.gallisepticum* over three time periods. Inoculation was on Day 10

	TIME INTERVAL		
GROUP	DAY 6-13	DAY 6-20	DAY 13-20
1 (non-infected)	59.70^{a}	152.10^{a}	92.40^{a}
2	64.80^{b}	130.90^{c}	66.10^{b}
3	57.20^{a}	142.90^{b}	85.75^{a}
4	57.85^{a}	146.30^{b}	88.40^{a}
5	57.60^{a}	143.90^{b}	86.30^{a}
6	56.00^{a}	143.20^{b}	87.40^{a}

N.B. Each group consisted of six replicated pens of 10 chickens

Superscripts denote significant differences down the columns for each time interval ($p \leq 0.05$)

Post mortem **examinations (see Table 4)**

Summarised and rank data are presented in Tables 8 and 9 and mean lesion scores for each group are presented in Table 5, respectively. There was a clear correlation between the presence of airsac and peritonitis lesions, this is illustrated by Figure 7, and tested to be significant Table 10.

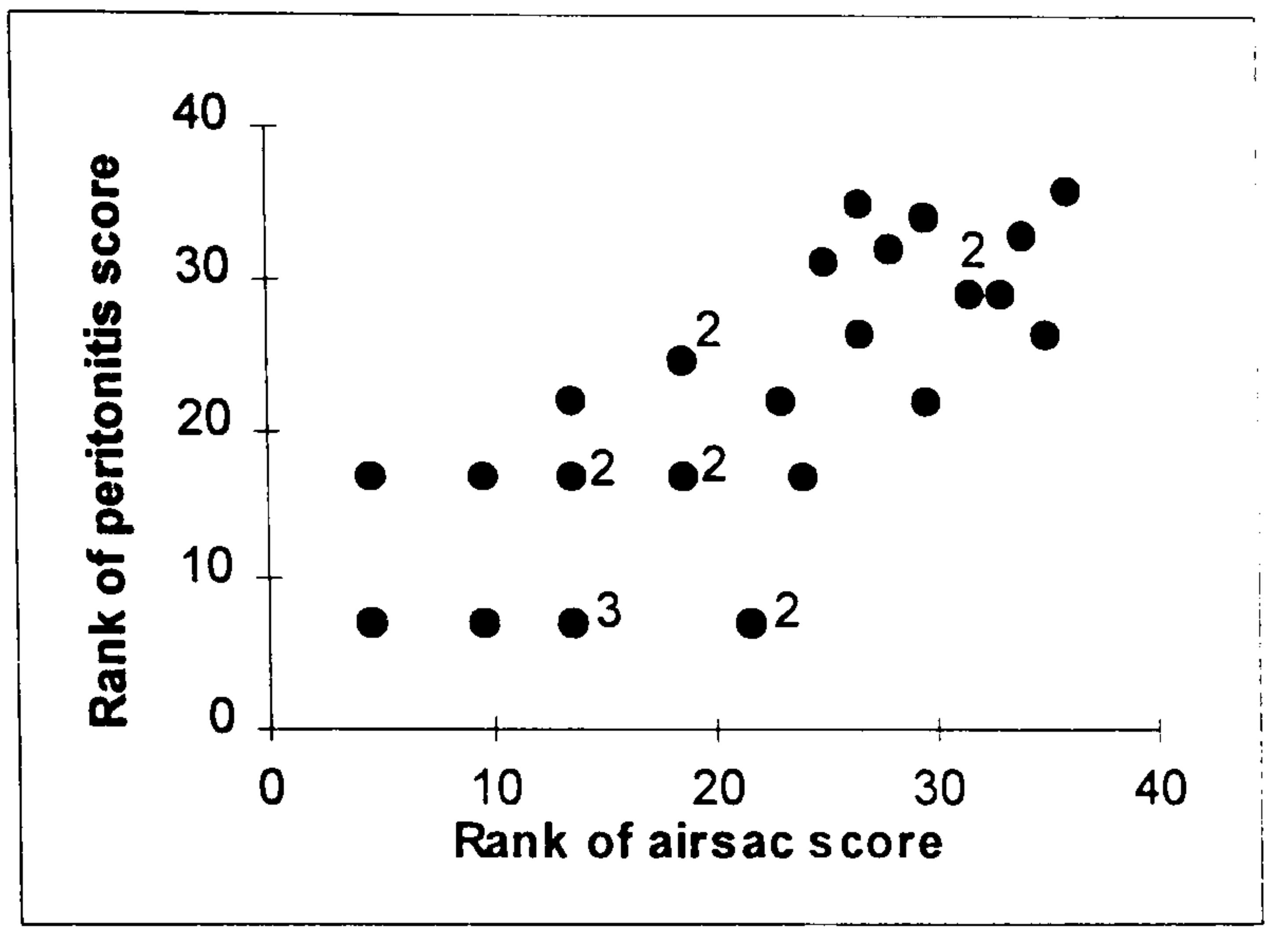
No airsac or peritonitis lesions were observed in control (non-infected/ sham-inoculated) chickens. Statistical analysis indicated that birds from all infected groups had significantly (p=0.05) more severe airsac lesions and peritonitis lesions than control birds.

Table 5: Mean airsac and peritonitis scores for six groups of 60 chickens grouped according to the severity of respiratory signs three days after experimental infection with *M.gallisepticum*. Necropsy was performed on all birds ten days after infection (standard errors are given in brackets)

GROUP	Airsacs	Peritonitis
1 (non-infected)	0.00 (0)	0.00 (0)
2	1.63 (0.5)	1.28 (0.6)
3	1.55 (0.6)	0.62 (0.3)
4	0.52 (0.3)	0.27 (0.3)
5	0.23 (0.1)	0.05 (0.1)
6	0.15 (0.1)	0.07 (0.1)

N.B. Each group consisted of six replicated pens of 10 chickens

Figure 7: The relationship between ranked data (by pen) for peritonitis and airsac scores for the 30 pens of 10 chickens which were infected with *M.gallisepticum*



N.B. Numbers next to plotted points indicate the number of identical values

Mycoplasma recovery (Table 6)

No mycoplasma were recovered from non-infected birds. Mycoplasma were recovered from 70 of the 300 birds (23.3 percent) in the experimentally infected groups.

Table 6: Number of positive *Mycoplasma gallisepticum* samples / number of tested samples from six groups of 60 experimentally-infected chickens

GROUP	T0	T-1	T-2
1 (non-infected)	0/60*	0/60	0/60
2	60/60	60/60	60/60
3	8/60	18/60	40/60
4	2/60	6/60	15/60
5	0/60	0/60	1/60
6	0/60	0/60	0/60

N.B. Each group consisted of six replicated pens of 10 chickens

T-1 and T-2 were ten fold dilutions from the TO seeder tube (see Appendix). The serial dilution increased the sensitivity of the test

Mycoplasma serology

In the 20 chickens blood sampled on Day 0, no maternal antibodies to *M.gallisepticum* were found. The number and percentage of seropositive birds ten days after infection is given in Table 7.

Table 7: Seroprevalence of *M.gallisepticum* ten days after experimental infection (day 20)

GROUP		
1 (non-infected)	0/60	0%
2	46/58	79%
3	24/60	40%
4	4/60	7%
5	0/60	0%
6	0/57	0%

Overview of results

Five birds died during the course of the study, there was no particular group disposition and these were considered within the normal bounds of 'incidental' mortalities expected within a group of this size (i.e. <1.5%). A few days after infection, birds showed respiratory signs and the intensity increased until last observation day. The mean bodyweight and the mean water consumption of infected birds was decreased compared to non-infected ones, however a decrease in water intake was also noted after sham-inoculation. This is presumably due to the stress to the birds of being handled and the inoculation procedure. A similar level of stress would be caused by parenteral vaccination. Lesions of air sacs and peritoneum were observed in 67 percent and 58 percent, respectively, of 300 *M.gallisepticum* inoculated chickens. *M.gallisepticum* could be reisolated from 100 percent of birds in the most severely affected group of 6 pens (mean airsac score 1.63, mean peritonitis score 1.28). According to serum agglutination tests, 79.3 percent of birds were positive in the most severely affected 6 pen group (mean score 1.63 for airsacs and 1.28 for peritonitis); 24/60 were positive for birds from the second most severely affected band of 6 pens (mean airsac score 1.55; peritonitis score 0.62); for the fourth group only 4/60 were seropositive (airsac score 0.52; peritonitis score 0.27). No seropositivity to *Mycoplasma gallisepticum* was detected for the remaining two groups of 6 pens each with lower mean scores (airsac scores 0.23 and 0.15 and peritonitis scores of 0.05 and 0.07). This may indicate a failure of infection at inoculation.

Five variables from the study were available for statistical analysis:

Airsac score
Respiratory score
Peritonitis score
Percent mycoplasma reisolation
Percent mycoplasma seropositive

Six pens were allocated to each group, with ten chickens in each pen. The pen has been taken as the statistical unit, and so where possible the scores used in the correlation analysis were the pen means, i.e. 36 mean values were used. However, percent mycoplasma reisolation and seroprevalence were only recorded for each group, and so these parameters have been correlated with the group means of the other three variables.

The results presented consist of correlation coefficients and p-values for each pair of variables. In each case, Spearman's correlation coefficient was calculated, i.e. given n pairs of observations (x_i , y_i) (i=1,2,...,n) for two variables x and y the coefficient is:

$$\theta = \frac{\sum_{i=1}^{n}(r_i - \bar{r})(s_i - \bar{s})}{\sqrt{\sum_{i=1}^{n}(r_i - \bar{r})^2 \sum_{i=1}^{n}(s_i - \bar{s})^2}}$$

where r_i denotes the rank of x_i, s_i denotes the rank of y_i, and $\bar{r}$ and $\bar{s}$ denote the mean values of the ranks of x and y respectively. The p-value was obtained by calculating the probability of obtaining a correlation at least as big as the one observed for the given number of observations, under the hypothesis of there being no relationship between the two variables. This was done automatically using the CORR procedure in the SAS for Windows (Version 6.12) package.

Table 8: Clinical and pathological data and ranked data (by pen replicates) for chickens experimentally inoculated with *Mycoplasma gallisepticum*.

Chicken pen data			RANKS	RANKS	RANKS
Airsac score	Respiratory score	Peritonitis score	Airsac score	Respiratory score	Peritonitis score
0	0	0	4.5	3.5	7
0	0	0	4.5	3.5	7
0	0	0	4.5	3.5	7
0	0	0	4.5	3.5	7
0	0	0	4.5	3.5	7
0	0	0	4.5	3.5	7
1.5	1.8	1.3	29.5	33	34
1.2	1.6	1.4	26.5	31	35
2.7	2	2.6	36	35.5	36
1.4	1.8	1.1	28	33	32
1.2	1.8	0.6	26.5	33	26.5
1.8	2	0.7	33	35.5	29
1.5	0.4	0.2	29.5	19.5	22
0.3	0.4	0.3	18.5	19.5	24.5
1.7	0.2	0.7	31.5	10.5	29
1.7	0.1	0.7	31.5	8	29
2.2	0.3	0.6	35	13	26.5
1.9	0.4	1.2	34	19.5	33
0.8	0.4	0.1	24	19.5	17
0.1	0.3	0.1	9.5	13	17
0.9	0.4	0.9	25	19.5	31
0.6	0.4	0.2	23	19.5	22
0.4	0.4	0	21.5	19.5	7
0.3	0.1	0.3	18.5	8	24.5
0.2	0.4	0.2	13.5	19.5	22
0.4	0.2	0	21.5	10.5	7
0.2	0.6	0	13.5	28.5	7
0.3	0.4	0.1	18.5	19.5	17
0.2	0.1	0	13.5	8	7
0.1	0.6	0	9.5	28.5	7
0	0.3	0.1	4.5	13	17
0.2	0.4	0.1	13.5	19.5	17
0.3	0.5	0.1	18.5	26	17
0.2	0.7	0.1	13.5	30	17
0.2	0.5	0	13.5	26	7
0	0.5	0	4.5	26	7

Table 9: Clinical and pathological data and ranked data (by severity banded groups) for chickens experimentally inoculated with *Mycoplasma gallisepticum*

Chicken group data					RANKS	RANKS	RANKS	RANKS	RANKS
Airsac score	Resp. score	Peritonitis score	*Mg* Reisoln (%)	*Mg* Sero-positive (%)	Airsac score	Resp. score	Peritonitis score	*Mg* Reisoln	*Mg* Sero-positive
0.00	0.00	0.00	0.00	0.00	1	1	1	1.5	2
1.63	1.83	1.28	100.00	79.31	6	6	6	6	6
1.55	0.30	0.62	66.67	40.00	5	2	5	5	5
0.52	0.33	0.27	25.00	6.67	4	3	4	4	4
0.23	0.38	0.05	1.67	0.00	3	4	2	3	2
0.15	0.48	0.07	0.00	0.00	2	5	3	1.5	2

In Table 10, correlations significant at the 5 percent level have been highlighted in bold. For the pen data all the correlations between airsac score, respiratory score and peritonitis score were significant, although the correlations involving respiratory score were not very strong. For the grouped data, all the correlations were significant except those involving respiratory score. However, the small number of observations should be taken into account when considering the size of the correlation coefficients.

Table 10: Correlation of clinical and pathological scores in chickens experimentally infected with *Mycoplasma gallisepticum*

	Correlation coefficient (p-value)	Airsac score	Respiratory score	Peritonitis score	% Mycoplasma reisolation
Pen data (n=36)	Respiratory score	**0.48 (0.003)**	-	-	-
	Peritonitis score	**0.82 (<0.001)**	**0.48 (0.003)**	-	-
Grouped data (n=6)	% Mycoplasma reisolation	**0.99 (<0.001)**	0.32 (0.54)	**0.90 (0.01)**	-
	% Mycoplasma seropositive	**0.94 (0.005)**	0.27 (0.60)	**0.94 (0.005)**	**0.95 (0.003)**

Discussion and conclusions

It was concluded that water consumption is not a parameter that can be used to quantatively grade the severity of disease. However, it can be of use to detect the presence of a number of physiological stressors upon the birds which may include disease. A decrease in water usage is one of the parameters used by commercial chicken producers to detect the outbreak of disease and is used by many production units as a trigger to commence preventive or metaphylactic medication programmes (Lister, S., 1996 - personnal communication). Based upon these data, although water consumption may be indicative of disease or another episode of stress (such as that induced by handling and sham-inoculation or vaccination), it does not appear to be useful as an indicator of disease severity.

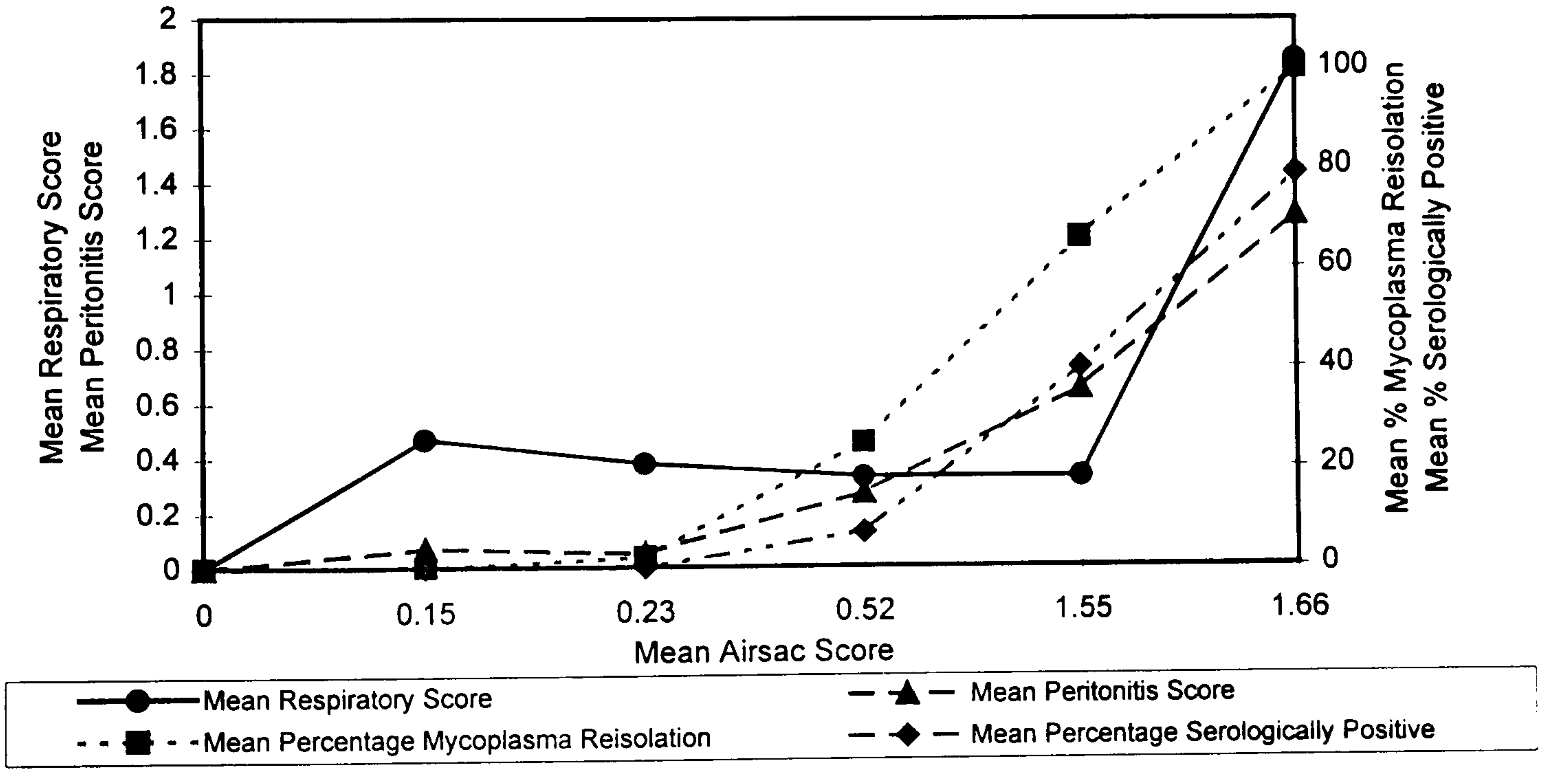
Growth was adversely affected by infection with *Mycoplasma gallisepticum*, for birds with the most severe clinical signs, bodyweight at the end of the 10 day study period was decreased by 11 percent. According to Kempf, I. (1998 -personal communication) the economic losses due to *M.gallisepticum* infection to producers over the whole grow-out period can be up to 20-30 percent reduction of weight gain, 10-20 percent decrease in feed conversion efficiency, 5-10 percent mortality and 10-20 percent condemnation of carcasses at processing. There was no significant difference (p=0.05) between the final bodyweights of groups which exhibited milder clinical signs. The mean bodyweight of groups 3-6 at the end of the study period was 4 percent below that of the sham-inoculated control birds (Table 3). Bodyweight would therefore seem to be of limited value as an indicator of disease severity, and only useful as a distinguishing criterion between severe and or very mild disease, but did not show a gradation in response. This could be related to the fact that until the physiological reserve of the respiratory system is compromised, there is little effect upon growth performance. The bodyweight gain data supports these findings (table 4), birds with severe clinical syptoms (group 2) showed bodyweight gains 28 percent lower than control birds (group 1) over the post-infection period (days 13-20), whilst there was no significant difference (p=0.05) between the mildly infected groups (3-6) and bodyweight gain was only 6 percent below the control birds. This study was only conducted over a 10 day period after infection and greater depression in bodyweight gains may become evident with more chronic disease. However, early indicators of disease are of more use to the commercial producer in order to instigate treatment before major economic losses occur.

Airsac lesions were closely correlated with clinical signs. However, as illustrated in Figure 8, before clinical signs becoming evident airsac lesions generally surpass a threshold of approximately 1.6. This too supports the hypothesis that there is a physiological reserve in the respiratory system which can be compromised before overt clinical signs are recorded. Peritonitis and airsac scores closely followed each other (Tables 5, 8 and 9), these parameters are strongly correlated with each other and with the clinical respiratory score (Table 10) indicating that they are good indicators of disease severity (Reeve-Johnson 1997). Although no other literature investigates the correlation between clinical signs and airsac or peritonitis lesion scores, the lesion scoring system, upon which the system used in this research is closely based, has been widely used since it was first described by Kleven *et al.*(1972). Recent examples include (Kempf *et al.* 1997, Reeve-Johnson *et al.* 1997a, Charleston *et al.* 1998). However, it can be seen from Table 5 that at low airsac and peritonitis lesion scores, the standard error approaches the mean, indicating the increased variability at low mean group levels when a few birds may have lesions and others none. This indicates that at low lesion scores, the scoring systems have less predictive value for the status of other birds in the group. At mean airsac lesion scores 1.63 and 1.55 the mean group levels were approximately three times the standard error (likewise for peritonitis score 1.28), at airsac lesion score 0.52 and peritonitis score 0.62, the standard error was approximately half the mean and at airsac lesion score 0.15 and peritonitis score 0.05 and 0.07 the standard errors was approxiamtely equal to the mean. Mycoplasma reisolations and seropositivity increased with worsening respiratory signs.

The statistical models are based upon linear correlations and this can be misleading because the clinical scoring systems do not give a linear representation of pathological progression. Although clinical scoring systems are designed to reflect a gradation in disease severity and progression, they have to be based upon signs which are distinguishable by the observer. The progression from not presenting a clinical sign to it becoming present may reflect either a small or alternatively a very large pathological progression. Consequently it is important when discussing clinical signs not to regard a doubling in the score (which is an arbitary gradation of clinical signs) as a doubling in pathological disease severity.

Table 10 shows the strong overall correlation between the indicators of disease severity investigated in this study, while Figure 8 is useful in illustrating that the relationship between the variables, whilst positively correlated, is not linear.

Figure 8: A graphical representation of the relationship between airsac score, peritonitis score, respiratory score, seroprevalence and microbiological reisolation rate in chickens infected with *M.gallisepticum*



Chapter 4: Experimental infection of pigs and the evaluation of disease severity

Introduction

In 1995 in Europe, there were 110.9 million pigs which accounted for 742 million ECU of animal health sales which was 23 percent of the total European animal health market. Forty-one percent of these animal health sales were antibacterial products (Anon 1997). Respiratory disease, involving *Actinobacillus pleuropneumoniae* is a cause of severe loss to the pig-rearing industry world-wide (Nicolet 1992). The disease is characterised by a haemorragic necrotising pneumonia and fibrinous pleuritis. Twelve serotypes of *A. pleuropneumoniae* have been described. The distribution of serotypes varies widely by geographic region.

A. pleuropneumoniae is a gram negative capsulated rod. Based on Nicotinamide-Adenosine-Dinucleotide (NAD) requirements, *A. pleuropneumoniae* can be divided into NAD-dependent (biotype 1) and NAD-independent (biotype 2) strains. Biotype 2 strains were formerly called *Pasteurella haemolytica* - like organisms, but in 1983, these strains were transferred to the genus *Actinobacillus* and combined with *Haemophilus pleuropneumoniae* into the new species *A. pleuropneumoniae* (Pohl *et al.* 1983, Niven and Levesque 1988). At present, 10 serotypes have been described in biotype 1 (serotype 5 is divided into a & b) (Neilsen 1986). Two serotypes have been described in biotype 2 (Fodor *et al.* 1989).

Although all serotypes cause disease, their virulence varies. Field observations and experimental infections provide evidence that biotype 2 strains are less virulent than biotype 1 strains, and it has been reported that, based on field observations, biotype 1 serotypes 1, 5a, 5b, 9 and 10 strains are more virulent than other biotype 1 serotypes (Dom and Haesebrouck 1992). This has not been confirmed under experimental conditions and the high pathogenicity of *A.pleuropneumonia* is considered to be due to a number of factors (Nicolet 1992).

It is generally accepted that the infective organism is inhaled directly into the lungs, however, using intravenous inoculation of a biotype 1 serotype 2 strain, the typical necrotising fibrinous pleuritis has been reproduced (Haesebrouck, F., 1998 - personal communication).

Actinobaccillosis is a major respiratory disease in the pig-rearing industry. The most important virulence factors of *A. pleuropneumoniae* are the Apx-toxins that lyse alveolar macrophages and polymorphonuclear cells (Kamp and van Leengoed 1989, Cruijsen *et al.* 1992). Thus *A. pleuropneumoniae* escapes from the primary defence of the host.

Three experimental infection models were used, each involving a different strain of *A. pleuropneumoniae*. A clinical scoring system was devised in order that a correlation between the clinical picture and pathology could be investigated for this variable condition. The same clinical scoring systems and pathological criteria were used in each study.

Materials and Methods

Landrace x Large White x Duroc pigs of minimal disease status were used. Animals were held under a natural day and night lighting regime in thermostatically regulated rooms maintained at $21 \pm 2°C$. The pigs were moved from the breeding herd to the animal facilities and housed in Multi Purpose Boxes (MPBs). These MPBs allow strict barrier conditions to be maintained between groups of animals by means of airtight rooms with a High Efficiency Particulate Air (HEPA) filtration system, negative pressure, airlocks where attendants change clothes and boots, and a vacuum circuit for manure removal to autoclaves. Rubber mats were placed on the concrete floor to provide a lying area. Each MPB was split into two separate pens. Pigs were identified by an ear-tag.

Before entering a MPB, workers changed overalls, boots, surgical masks, hats, and gloves. Before the studies, MPBs were cleaned and disinfected with formaldehyde. During the studies, boxes were cleaned daily with high pressure water. Directly after cleaning, the floor was wiped dry so that the pigs were not able to drink this water.

The health status of the animals was checked upon arrival. Health was judged on the basis of demeanour and absence of disease signs in particular of a respiratory, nervous and locomotory nature. Only healthy animals were used. Pigs were observed closely during the acclimatisation period to ensure adequate feed and water intake and for evidence of fighting, vices or incidental diseases. Pigs were observed at least twice daily throughout the study.

Drinking water fit for human consumption from the local drinking water company was available *ad libitum.* Water utilisation was measured with a water meter and spilled water was collected in catchment bowls beneath the floor slats. Water consumption was calculated by subtracting the spilled volume of water from the total utilisation for each group of pigs. The water intake of groups of pigs was monitored in each study to determine whether this could be used as an indicator of disease outbreak or a measure of disease severity.

A single batch of irradiated feed of standard formulation was used. This feed was prepared under Good Manufacturing Practice standards (GMP) and did not contain any antibiotics. The quantity of feed offered and quantity of remaining feed at the end of the study were recorded. Feed was provided daily during the morning at a rate of approximately 65 g feed per kg bodyweight.

Pigs were weighed using an electronic weighing system at intake, on the day before challenge and on the day of necropsy. The pre-intake weights were used to allocate animals to groups. Male and female pigs were separately ranked in order of weight and then randomly allocated to each group in sequence such that an even sex balance and approximately equal weight of pigs were obtained in each group.

Pigs were experimentally infected with *A. pleuropneumoniae* once they had acclimatised. In Study 1 and Study 2, pigs were infected with aerosolised *A. pleuropneumoniae* serotype 3 and 5a respectively. In Study 3, an endobronchial infection method was used to deliver serotype 9 to the level of the tracheal bifurcation in the lungs.

Clinical examinations

Pigs were examined for clinical signs after challenge infection twice daily for approximately ten days. Pigs were examined for welfare purposes more frequently as required. Pigs showing signs of moderate distress likely to progress or continue were euthanased and *post mortem* examinations performed. Moderate distress was defined as a clinical demeanour score of 3 on two consecutive examinations 12 hours apart or a single clinical demeanour score of 4.

Rectal temperatures were determined with an electronic thermometer in the morning for the two days preceding challenge in order that the pigs became habituated to the practice. On the following days, temperatures were determined in the morning and in the afternoon.

Scoring of clinical signs and pathological lesions caused by experimental *Actinobacillus pleuropneumoniae* infections in pigs

Emphasis was placed upon attempting to define a suitable scoring system for use in these three studies. Systems ranking clinical signs as 'mild', 'moderate' and 'severe', were felt to be too loose. Although experienced clinicians who regularly used such systems felt that they were reliable and a good reflection of clinical demeanour in their own hands, there was not enough information which could be transferred consistently to other individuals. Information transfer is a major objective of a scoring system and to maximise this a more detailed system was devised. Difficulty was also experienced with systems which were too detailed. Different diseases may exhibit the same signs but different signs may also be presented by the same disease. Likewise individual animals vary greatly in the disease signs that they present and the order in which they are presented during the course of a disease episode. The result was the following system for demeanour scoring (Reeve-Johnson, 1999).

Demeanour Score

0 (normal)	**Alert pig that responds to an observer entering the pen, carries out normal behaviour and evades handling**
1 (first signs of disease)	**Less active and alert than normal pig, exhibits first signs of disease, may cough, skin may be blotchy, but still evades handling**
2 (clearly discernible disease signs)	**Pig slow in evading handling, walks about two metres, does not exhibit many normal behaviour activities, it may lie down while observer is in the pen**
3 (very dull)	**Pig losing interest in the environment, may require stimulation to move, prefers to lie, may not stand for more than one or two minutes**
4 (severely diseased)	**Totally disinterested in the environment and no effort to move away from observer even upon stimulation, difficulty in standing, requires immediate euthanasia**

Respiratory score

It is very difficult to observe respiratory movements in a healthy pig, it is also not possible to auscultate a pig easily with a stethoscope without handling. The stress associated with restraining and handling pigs generally causes a large increase in respiratory rate. A scoring system was therefore designed to describe the increasing respiratory effort of a pneumonic pig, which can be performed by careful observation from a distance.

Respiratory score

0	**Normal, no obvious respiratory movements, even when pig moving about pen**
1	**Respiratory rate slightly increased - have to watch pig carefully to notice, when recumbent. Respiratory movements clearly evident when pig moving about pen**
2	**Respiratory rate obviously increased, respiratory movements evident when pig recumbent. Abdominal respiratory movements clearly evident when pig moving about pen**
3	**Respiratory rate rapid at rest with pronounced abdominal respiratory phase. Dyspnoea immediately evident if pig forced to move in pen**

In one study (PNE9501) a cough score was also used:

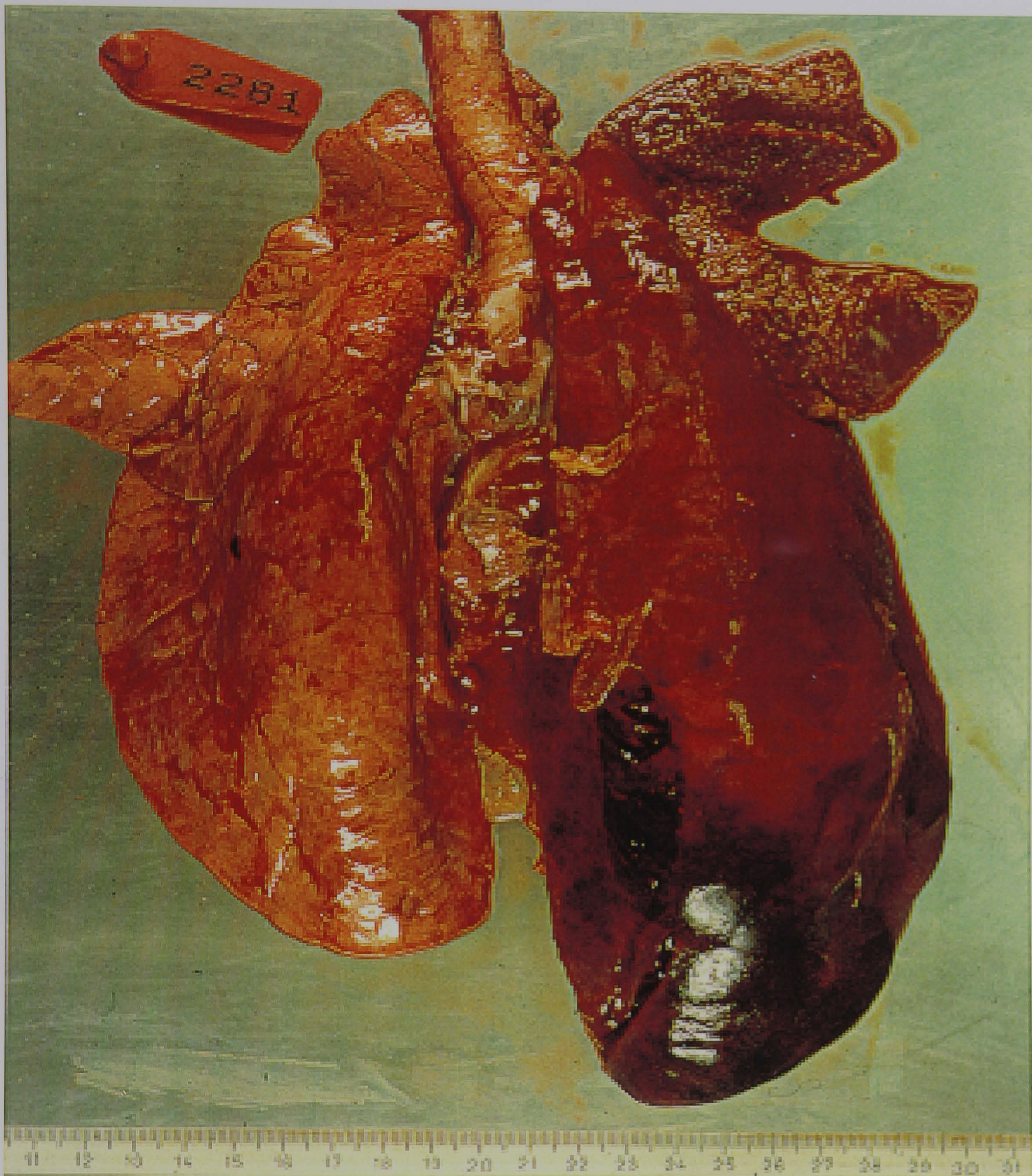
0	**No coughing within five minutes of observation or when observer enters pen and makes pig move**
1	**Coughing only when pig made to move**
2	**Pig coughs once or twice during 5 minute observation and becomes worse once pig made to move**
3	**Frequent coughing in resting pig**

Post mortem examinations

Pigs were euthanased with sodium pentobarbital (15 mg/kg) intravenously and exsanguinated by section of the carotid artery. Abdomen and thorax were opened and examined. Heart, liver, spleen, kidney were grossly examined and abnormalities were recorded. Lungs and trachea were taken out of the thorax, heart, larynx and all parts not belonging to the respiratory tract were trimmed off and then the lungs were weighed. Each lobe was examined visually and by palpation. The percentage of lung affected was estimated and recorded and the sites of pleuritis and lung lesions were recorded on a lung map. The lung lesions were cut with a knife and the tissue was swabbed with a cotton swab for bacteriological examination. Swab samples were taken from lesions of pneumonia (1 per pig) and from affected joints or periarticular tissues (1 per pig) for bacteriology. When no lung lesions were present, samples for bacteriology were taken from the middle of the lung lobes. The nature of the lung lesions based on clearly visible gross changes were recorded as:

catarrhal:	grey-red discoloration of the consolidated parenchyma
haemorrhagic:	parenchymal haemorrhages
necrotic:	tissue necrosis
fibrinous:	fibrinous dilatation of interlobular septae, dry, red appearance of the cut surface
abscess:	abscess surrounded by a fibrous capsule
fibrosis:	connective tissue formation

Figure 1: Typical *Actinobacillus pleuropneumoniae* infected lungs sampled at time of necropsy (serotype 3)



Note the fibrous adhesions on the pleural surface and with consolidation and congestion of the right lobe. The caudo-dorsal distribution of lung lesions of this example is typical of *Actinobacillus pleuropneumoniae* infection as opposed to the cranio-ventral distribution of lesions more typical of *Mycoplasma spp*. infections.

In one study (PNE9501), hyperplastic changes in the thoracic, sternal and tracheo-bronchial lymph nodes were recorded. The lymph nodes were examined and enlargement was recorded as:

Lymph node score

0	normal size
1	mildly enlarged (enlarged by less than 1/3)
2	moderately enlarged (enlarged by 1/3 or more, but less than double)
3	severely enlarged (enlarged by twice or more the normal size)

Bacteriology

The samples of nostrils, tonsils and lung tissues collected at necropsy were cultured on Columbia agar supplemented with 5% blood with a *Staphylococcus intermedius* inoculation streak. Identification of the colonies as *A. pleuropneumoniae* was based on the satellitism phenomenon, the positive haemolysis and the positive CAMP reaction.

Study 1 PUK9501:

Overview

Five groups, each of 12 pigs, 16-27 kg bodyweight were challenged with *Actinobacillus pleuropneumoniae* (serotype 3) by intranasal spray, 9 x 10^5 cfu per pig. Each group of 12 pigs was kept in the same room, but split into two identical replicated pens of 6 pigs each. All pigs in a single group were sequentially infected before pigs in the next group were inoculated. Because of the intimate contact of pigs with others in the group and the natural route of infection of *A. pleuropneumoniae* is by aerosol and direct contact, the statistical unit could be considered to be the group in a single airspace (i.e. 12 pigs). In the results section for the purposes of correlating the clinical data with pathological data, individual pig data was used, while for measurements of water consumption and feed intakes because pigs were kept in groups, group data had to be used. Pigs were monitored for clinical signs, raised body temperature, bodyweight changes and food intake for 14 days after experimental infection. Clinical scores were calculated from the observations of demeanour and respiratory abnormalities. Prior to challenge infection, rectal temperatures ranged from 38.7°C to 40.5°C. High values in apparently normal pigs are commonly recorded and are assumed to be a response to handling. Animals were considered febrile if the rectal temperatures were more than 2 standard deviations above the mean for normal pigs (all records, Days 8-10, 39.5 ± 0.42°C), i.e. ≥ 40.3°C. Surviving pigs were euthanased for *post mortem* examination 14-15 days after challenge and examined for lesions of pneumonia and presence of the challenge organism. The terminal clinical scores and body temperatures in addition to the bodyweight and growth parameters were then tested for statistical correlation with each other, and with the gross pathological signs of disease recorded *post mortem*. These parameters were plotted on graphs to illustrate the relationships between the variables. The major events in the study are summmarised in the following 'schedule of events'.

Challenge inoculum:

Organism	*Actinobacillus pleuropneumoniae*
Strain and serotype	K669, Serotype 3
Source	Pneumonic pig lung, isolated at MAFF Veterinary Investigation centre Bury St Edmunds, Suffolk, UK. February 1994
Pass number	Low passage (< 6 passages)
Storage details	Ultracold deep freeze (-78°C approximately)
Culture details	Broth culture
Challenge dose	9 x 10^5 colony-forming units (cfu) per pig
Dose reconciliation	Dose reconciliation by volume was performed
Dose volume	3 ml per pig
Administration	Intranasal spray application, approximately equal quantities in each nostril

Schedule of events:

Day 1	Introduce 60 Specific Pathogen Free Pigs Commence feeding antibiotic-free feed and maintain feed intake records.
Days 1-5	Allow pigs to acclimatize
Day 6	Commence twice daily records of water intake
Day 8	Take rectal temperatures once
Day 9	Take rectal temperatures once, weigh pigs
Day 10	Challenge pigs (afternoon) Take rectal temperature once
Day 11	Commence frequent clinical observations. Commence twice daily rectal temperatures. Emergency *post mortem* examinations if necessary (to end of study)
Day 12	Weigh pigs
Day 14	Cease records of water intake
Day 16	Weigh pigs
Day 17	Last rectal temperature
Day 18	Start single daily clinical examinations
Day 23	Weigh pigs
Day 24	Last clinical examination Weigh and necropsy half surviving pigs in each group
Day 25	Weigh and necropsy all remaining pigs

Results

Challenge infection produced moderately severe disease. Clinical signs including increased clinical demeanour and respiratory scores, sneezing and coughing were seen commencing 17 hours after challenge. All pigs became ill, with pronounced illness in seven animals and clinical signs persisting for 14 days in eight animals. Two pigs were euthanased within 30 hours of infection, lung lesions extended to 33 percent and 53 percent of the lung area. One was euthanased for humane reasons nine days after challenge. Marked pneumonia, affecting 23 percent of lung area, was observed in this pig at *post mortem* examination. The remaining pigs survived to the end of the study period and modest lesions of pneumonia affecting from 1-5 percent of lung area was observed. The clinical observations are summarized in Tables 1 and 2.

Intake of water

Water intakes on Day 11 for the period commencing approximately 30 hours after infection were reduced by approximately 40 percent in comparison to the previous 24 hours. Water intakes recovered after Day 11. The post-infection water intake in group 4 was half that of the other groups, group 4 was the median group in terms of both clinical scores and percentage lung consolidation upon pathological investigation. There was no significant difference between the water intakes of the other groups of pigs. The depression in water intake does not appear to have any direct relationship with overt clinical signs or gross pathology observed upon necropsy.

Table 1: Summary of clinical observations of five groups of 12 pigs experimentally infected with *A. pleropneumoniae* serotype 3

Group	Number and percentage of pigs (ex. 12) showing:			
	Intermittent disease signs	Prolonged disease signs	Mortality	No significant illness
1	10 (83%)	8 (66%)	0 (0)	2 (17%)
2	9 (75%)	6 (50%)	1 (8%)	0 (0)
3	7 (58%)	2 (17%)	1 (8%)	1 (8%)
4	9 (75%)	6 (50%)	0 (0)	1 (8%)
5	11 (92%)	7 (58%)	1 (8%)	0 (0)

Intermittent disease signs: demeanour score of at least 2 on at least one occassion

Prolonged diseased signs: demeanour score of 2 on at least 4 days in any period of 7 days; excludes pigs that required euthanasia

Mortality: pig required euthanasia prior to end of the study

Table 2: Summary of clinical scores for five groups of 12 pigs experimentally infected with *A. pleuropneumoniae* serotype 3

	Clinical scores [means and Standard Error ; median values in brackets]	
	Total study period*	
Group	Demeanour	Respiratory signs
1	16.6 ± 9.1 (18.5)	20.8 ± 12.7 (23.0)
2	12.4 ± 5.9 (14.0)	19.0 ± 8.2 (17.0)
3	10.4 ± 6.4 (11.0)	14.0 ± 9.0 (14.0)
4	13.5 ± 7.9 (13.5)	17.6 ± 9.5 (19.0)
5	11.6 ± 6.3 (13.0)	14.8 ± 5.3 *(15.0)*

N.B. n=12 pigs per group.
* Mean of total scores for pigs in each group recorded daily for 14 days post-challenge

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Rectal temperatures

Mean rectal temperatures rose to a peak on the afternoon of Day 11 (24 hours after experimental infection) with the majority of pigs in all groups being febrile. Temperatures fell rapidly after Day 11. The pattern of the temperature responses was not significantly different in any group (Kruskal-Wallis test: $p > 0.05$).

Bodyweight changes

Mean group bodyweight is displayed in Table 3. Bodyweight values for pigs that required euthanasia before the end of the study were excluded from summary data. The large standard errors quoted in Tables 2 and 3 for daily liveweight gain after infection, illustrated the differing impact that disease had on individual pigs. Despite having pigs of a similar age, weight and genetic background, infected with the same pathogen under the same experimental conditions, in some pigs there was a far greater negative impact on growth than in others. Although there were no pre-experiment liveweight gain data available, the pigs were approximately the same age and weight at the start of the study. This can be taken as evidence of similar individual liveweight gains prior to the study. The standard error of mean daily liveweight gain for the period Day 9-12 was greater for each group than the standard deviation over the whole period of Day 9-23. This illustrates that the acute phase of disease had a greater impact on growth uniformity than the later more chronic phase. In the inital phase of disease, some pigs exhibited weight loss, while others continued growing at approximately their pre-infection growth rate. This was at least partly due to the observed inappetance of some pigs and apparently normal appetites of others during this phase. From Day 12 after infection, all pigs were were growing again.

The bodyweight changes between Days 9 and 12 represent the acute loss in weight or growth check associated with inappetance (Table 3). Daily liveweight gains from Day 9 to Day 12 were lowest in Group 2 (Mean 0.06 ± 0.62 kg/day, median 0.17) and highest in Group 3 (mean 0.41 ± 0.40 kg/day, median 0.50). Differences between groups were not statistically significant (Kruskal-Wallis test, $p = 0.65$).

The bodyweight changes between Days 9 and 23 represent the effect of the induced disease on growth rate. The performances of the different groups were different, ranging from a mean of 0.84 ± 0.07 kg/day (median 0.82) for Group 3 to a mean of 0.66 ± 0.08 kg/day (median 0.64) for Group 5. Data were not normally distributed and non-parametric tests were performed. The overall distribution of results was significantly different from random distribution (Kruskal-Wallis test, $p = 0.008$).

Four group to group comparisons were significantly different: Group 1 to 3 ($p < 0.05$), Group 2 to 5 ($p < 0.02$), Group 3 to 4 ($p < 0.05$) and 3 to 5 ($p < 0.001$), using Mann-Whitney tests.

Table 3: Summary of bodyweight changes and food conversion ratios for five groups of 12 pigs experimentally infected with *A. pleuropneumoniae* Serotype 3

Group	Daily liveweight gain (kg/day) [Means and Standard Errors, medians in brackets] Day 9-12	Day 9-23	Food conversion ratio
1	0.10 ± 0.80 (0.25)	0.67 ± 0.23 (0.73)	1.89
2	0.06 ± 0.62 (0.17)	0.81 ± 0.15 (0.82)	1.72
3	0.41 ± 0.40 (0.50)	0.84 ± 0.07 (0.82)	1.89
4	0.36 ± 0.59 (0.33)	0.72 ± 0.24 (0.70)	1.87
5	0.14 ± 0.63 (0.33)	0.66 ± 0.08 (0.64)	2.03

Feed intake and food conversion ratios

The food conversion ratios (FCRs) are presented in Table 3.

Necropsy findings

Individual lung maps were used to record gross pathological changes.

Marked multiple lesions of acute pleuropneumonia with extensive lung oedema were observed in pigs euthanased within 30 hours of challenge infection (Pig Nos. 13 and 32). The lesions extended to 53 percent and 33 percent of the lung area, respectively. Lung-to-bodyweight ratios were high (in percentage terms: 2.9% and 1.82% respectively, normal < 1.06%) reflecting the extent of the lesions. Pig 57 was euthanased 9 days after challenge and had 23 percent of lung area affected with scattered foci of pleuropneumonia showing some chronic changes, with fibrosis, adhesion and necrotic centres. The lung-to-bodyweight ratio was 1.31 percent. Marked lesions of tenosynovitis with surrounding oedema affecting the elbows and hocks were observed.

A variety of pneumonic lesions were observed during *post mortem* examination of the lungs of the pigs that survived to the end of the study. Lesions were of chronic pleuropneumonia with fibrosis, caseous necrotic centres and pleuritic adhesions which affected up to 55 percent of lung area. There was marked variation in the area of lungs affected in all groups, many pigs having little or no pneumonia involvement. Seven pigs in Group 5 , had lesions comprising greater than 1 percent of lung area, but only one pig had >5 percent of lung area affected. Four, five, two and four pigs had more than 5 percent of lung involvement in Groups 1-4, respectively (Table 4).

Table 4: Summary of distribution of lesions over the total lung area at necropsy, 14 to 15 days after experimental infection with *A.pleuropneumoniae* serotype 3, for 58 pigs

Group	Number of pigs with percent pneumonia in range:			
	0-1%	1.1-5%	5.1-15%	>15%
1	6	2	0	4
2	2	4	3	2*
3	6	3	2	0*
4	6	2	2	2
5	4	6	1	1

N.B. n = 12 pigs per group.
*Pigs requiring euthanasia within 30 hours of challenge infection (one in each of Groups 2 and 3) excluded from analysis

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The percent pneumonia calculated for each pig, including those that required euthanasia prior to the end of the study, were analysed statistically. The data were not normally distributed (hence the large range) so non-parametric tests were performed. The distribution of the data were not significantly different from random distribution (Kruskal-Wallis test, $p = 0.54$)

Median lung-to-bodyweight ratios reflected the values for percent pneumonia; mean values were distorted by individual pigs with high values in Groups 1-3 (Table 5).

Table 5: Summary of necropsy results for 60 pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 3

Group	Lung lesion measurements [Means and Standard Error, medians in brackets]		Number culture positive**
	% pneumonia	LBWtR (%)*	
1	11.1 ± 17.0 (1.3)	1.20 ± 0.44 (1.06)	6/12
2	11.9 ± 16.3 (4.6)	1.27 ± 0.54 (1.13)	7/12
3	4.6 ± 9.2 (1.6)	1.09 ± 0.25 *(1.05)*	3/12
4	10.0 ± 17.6 (1.2)	1.14 ± 0.32 (1.11)	3/12
5	4.6 ± 6.6 (2.4)	1.09 ± 0.10 (1.07)	4/12

*Lung-to-bodyweight ratio

**Number of pigs positive for *A. pleuropneumoniae* in lung tissue (12 pigs per group)

Bacteriology

A. pleuropneumoniae was recovered from the lungs of the majority of pigs with >7 percent pneumonia involvement. The number of pigs in each group from which positive cultures of *A. pleuropneumoniae* were obtained is summarized in Table 5.

A summary of the clinical and pathological data is contained in Table 6.

Table 6: Clinical, pathological data and ranked data for pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 3

PUK9501 Pig no.	Clinical score	Rectal Temp. (°C)	Resp. score	Lung consoln. (%)	Lung/ Bwt ratio	RANKS Clinical score	RANKS Rectal Temp. (°C)	RANKS Resp. score	RANKS Lung consoln. (%)	RANKS Lung/ Bwt ratio
2	0	39.9	0	25	1.35	19	49	17.5	52	53
5	0	39.5	0	0	0.85	19	27	17.5	5.5	2.5
6	0	39.8	0	0.4	0.94	19	45	17.5	14	8.5
8	0	39.5	1	0.1	1.03	19	27	43.5	11	22
10	0	40	3	54	2.5	19	54	58	57	57
11	0	39	1	0	0.98	19	4.5	43.5	5.5	16
12	0	39.4	0	0.5	1.06	19	17	17.5	18	27.5
15	0	39.9	0	33	1.41	19	49	17.5	54	55
16	0	39.5	1	11	1.07	19	27	43.5	47.5	32
17	0	39.3	0	9	1.12	19	11	17.5	46	37
18	0	39.5	0	0.8	0.9	19	27	17.5	24	5.5
19	0	39.9	1	1.8	1.31	19	49	43.5	27.5	52
20	0	39.2	0	3.9	1.04	19	7.5	17.5	36	24
21	0	40	2	5.4	1.3	19	54	55	43	51
24	0	40	0	1.8	0.97	19	54	17.5	27.5	13
25	0	39.6	0	0.5	1.06	19	36.5	17.5	18	27.5
26	0	39.5	0	0	1.01	19	27	17.5	5.5	18.5
27	0	39.7	0	0.4	1.04	19	41.5	17.5	14	24
28	0	39.5	0	0	0.85	19	27	17.5	5.5	2.5
33	0	39	0	6.3	1.07	19	4.5	17.5	44	32
36	0	39.6	0	2.5	1.21	19	36.5	17.5	31.5	48.5
40	0	39.2	0	5.3	1.14	19	7.5	17.5	42	40.5
41	0	39.7	0	0	1.14	19	41.5	17.5	5.5	40.5
44	0	38.8	0	14	1.16	19	3	17.5	49	45
45	0	39.8	0	49	2.04	19	45	17.5	56	56
46	0	39.3	0	0.4	0.8	19	11	17.5	14	1
47	0	39.1	0	1.6	0.97	19	6	17.5	26	13
48	0	39.5	0	0.5	0.98	19	27	17.5	18	16
49	0	39.4	0	0	0.98	19	17	17.5	5.5	16
50	0	40.1	0	0.4	1.02	19	57	17.5	14	20.5
51	0	39.5	0	4.6	1.14	19	27	17.5	40	40.5
52	0	40	0	11	1.17	19	54	17.5	47.5	47
53	0	39.6	1	0.4	1.08	19	36.5	43.5	14	35
55	0	39.5	1	0.7	1.16	19	27	43.5	22	45
56	0	39.6	0	4.2	1.01	19	36.5	17.5	37	18.5
58	0	39.9	0	2.5	1.04	19	49	17.5	31.5	24
59	0	39.7	0	2.3	0.97	19	41.5	17.5	30	13
1	1	39.5	1	3.6	1.21	45	27	43.5	35	48.5
4	1	39.9	1	27	1.27	45	49	43.5	53	50
7	1	39.5	1	2	0.95	45	27	43.5	29	10.5
9	1	39.5	1	21	1.16	45	27	43.5	51	45
14	1	39.4	0	2.6	1.15	45	17	17.5	33.5	43
23	1	39.6	2	0	0.9	45	36.5	55	5.5	5.5
29	1	39.5	1	7	0.95	45	27	43.5	45	10.5
31	1	38.7	2	4.3	1.07	45	2	55	38	32
34	1	39.4	1	2.6	1.07	45	17	43.5	33.5	32
35	1	39.3	0	0	0.93	45	11	17.5	5.5	7
38	1	39.3	0	4.7	1.14	45	11	17.5	41	40.5
42	1	39.4	1	0	1.02	45	17	43.5	5.5	20.5

43	1	40	1	44	1.37	45	54	43.5	55	54
54	1	39.7	1	1.5	1.06	45	41.5	43.5	25	27.5
60	1	39.4	0	4.5	1.09	45	17	17.5	39	36
3	2	39.4	1	0.6	1.06	55.5	17	43.5	20	27.5
22	2	38.5	2	20	1.13	55.5	1	55	50	38
30	2	39.3	0	0.7	0.94	55.5	11	17.5	22	8.5
37	2	39.8	1	0	0.88	55.5	45	43.5	5.5	4
39	2	39.6	2	0.7	1.07	55.5	36.5	55	22	32
57	2	40.8	1	.	.	55.5	58	43.5	.	.

N.B. consoln. = consolidation; Bwt = Bodyweight

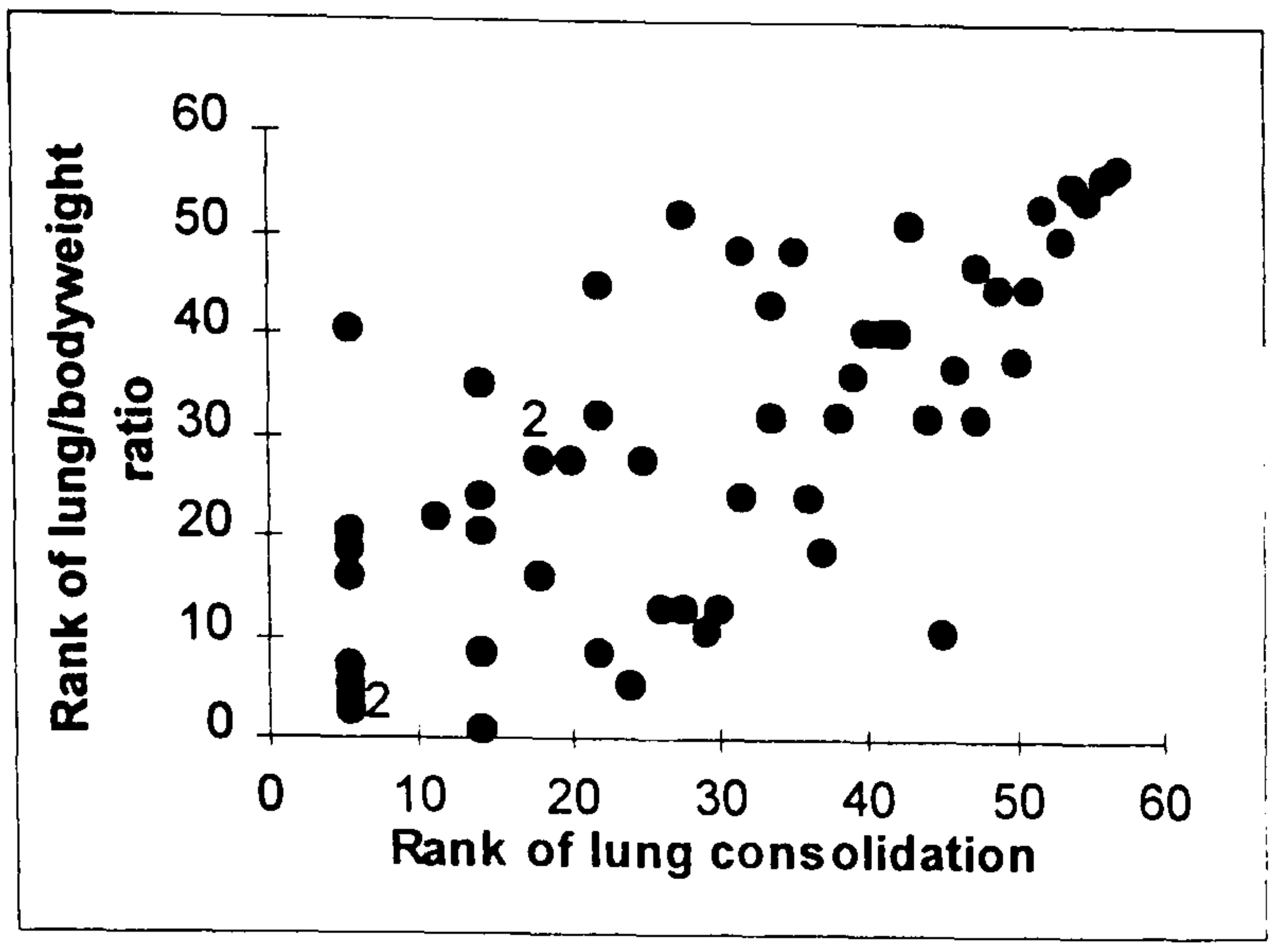
Two highly significant correlations were noted, between clinical demeanour score and respiratory score (Table 8), and between lung consolidation and lung/ bodyweight ratio ($p<0.001$) (Figure 2 and Table 8). It should be noted that for half the animals in this study, both the clinical demeanour score and the respiratory score were zero. However, a chi-square analysis of the two-way table of clinical demeanour score versus respiratory score, with both scores recorded as zero or non-zero, also provided highly significant evidence of an association between these two variables ($p=0.001$). None of the other pairwise correlations in this study were significant at the 5 percent level (Table 8).

Table 7: Two-way table showing numbers of animals/ respiratory score at each clinical demeanour score for 58 pigs experimentally infected with *A. pleuropneumoniae* serotype 3

number of animals	Demeanour score 0	1	2	3
Respiratory score				
0	29	4	1	0
1	6	9	3	0
2	1	2	2	**2**
3	1	0	0	0

N.B. the two pigs euthanased within 30 hours of infection are highlighted in bold type

Figure 2: Graphical relationship between ranked data for lung/ bodyweight ratio (expressed as %) and ranked lung consolidation data for 60 pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 3.



N.B. Numbers next to plotted points indicate the number of identical values.

Table 8: Spearman's correlation coefficients demonstrating the strength of correlation between rectal temperature, respiratory score, percent lung consolidation and lung: bodyweight ratio in 60 pigs experimentally infected with *Actinobacillus pleuropneumoniae* serotype 3

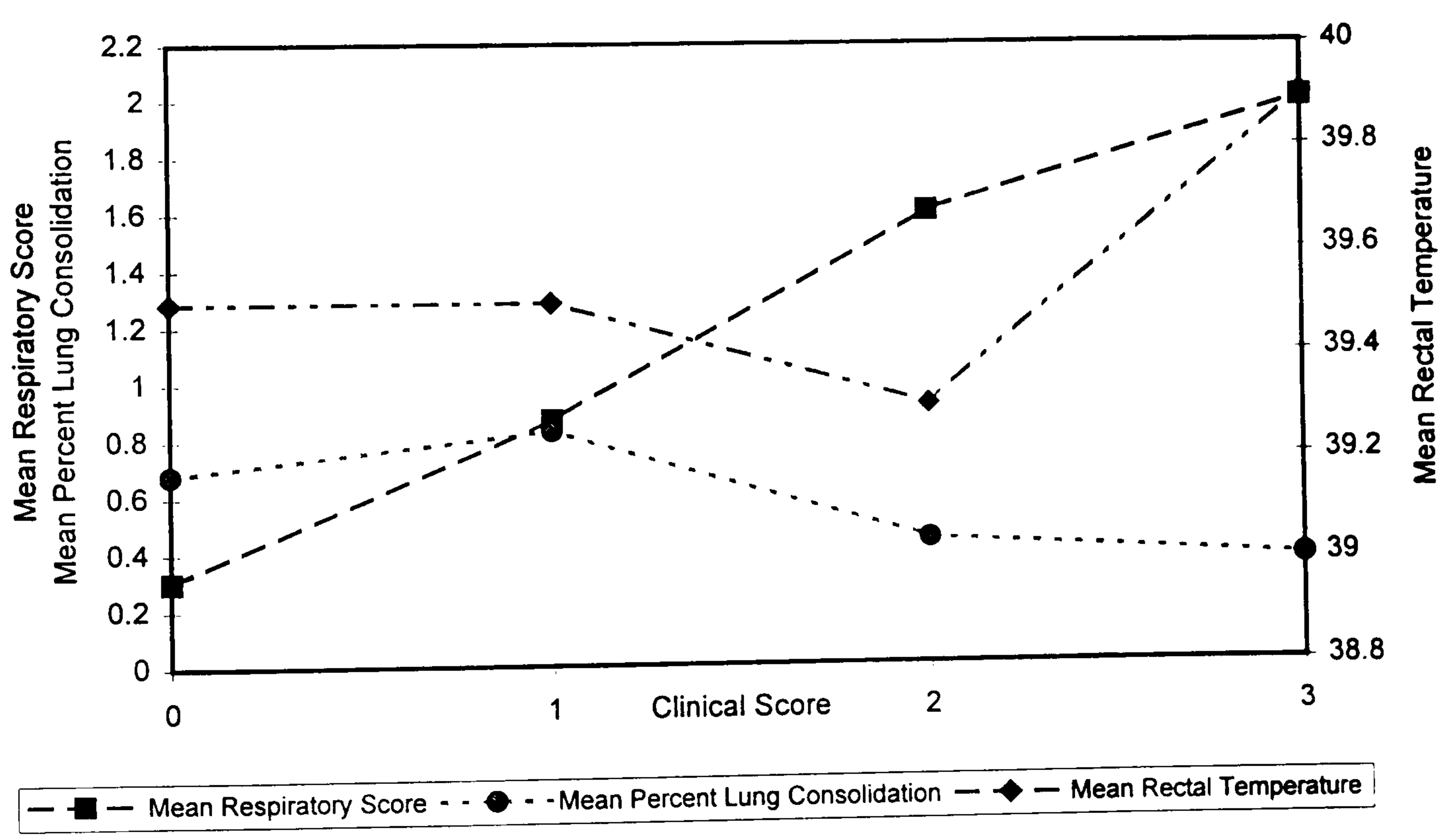
Study	Correlation coefficient (p-value)	Clinical score	Rectal temperature	Respiratory score	% Lung consolidation	Lung/bodyweight ratio (%)
PUK9501	Rectal temperature	-0.13 (0.30)	-	-	-	-
(n=58)	Respiratory score	**0.52 (<0.001)**	0.11 (0.41)	-	-	-
	% Lung consolidation	0.05 (0.72)	0.10 (0.45)	0.10 (0.44)	-	-
	Lung/bodyweight ratio (%)	-0.04 (0.76)	0.25 (0.06)	0.19 (0.15)	**0.71 (<0.001)**	-

N.B.

1. n=58 except for correlations involving percent lung consolidation and lung/ bodyweight ratio where n=57
2. numbers in bold denote statistically significant correlations, where $p<0.05$ (p values are given in brackets)

There was an insufficient range of clinical scores between the groups to correlate demeanour scores with production data such as Average Daily Feed Intake and Feed Conversion Ratio data. The median demeanour score for the total study period varied between 11.0 and 18.5 (Table 2) while the median Feed Conversion Ratio's varied between 1.72 and 2.03 (Table 3). Many more groups would be needed to correlate data which has been measured on a group basis, such as feed intake. Median values are probably more applicable for comparison of demeanour score over the entire study period than the means, because group mean values are influenced more by individual extreme cases and do not reflect the 'group' as well. There were insufficient data to evaluate the group effects on production data, and this is outwith the scope of this thesis, but it would make interesting additional research provided a sufficient number of replicated groups under similar conditions were available.

Figure 3: Graphical representation of the relationship between clinical demeanour score and respiratory score, percent lung consolidation and mean rectal temperature for pigs infected with *Actinobacillus pleuropneumoniae* serotype 3



Study 2: PBE9501

Overview

Forty eight-week-old pigs were divided at random into five groups of eight pigs. Each group of pigs was housed in a separate room. The pigs were inoculated intra-nasally with 10^7 cfu of an *A. pleuropneumoniae* serotype 5a strain. All pigs were examined daily for clinical disease. Clinical demeanour and respiratory signs were scored daily. Rectal temperatures were recorded daily. Pigs that died after infection were necropsied immediately. Pigs with severe respiratory distress, lethargy or weakness were euthanased. Surviving pigs were euthanased for *post mortem* examination 9-10 days after infection and examined for lung lesions and presence of *A. pleuropneumoniae* in the lung. Data were then examined for correlation between clinical demeanour and respiratory scores with each other and with rectal temperature, percent lung consolidation, and the lung to bodyweight ratio which was expressed as a percent.

Materials and methods

Forty pigs were obtained from a single herd with no history of Actinobacillosis. Blood samples were taken at three and five weeks-old and tested for antibodies against *A. pleuropneumoniae* RTX toxins ApxI and ApxII as described by Dom *et al.* (1994a), to ensure that there was no pre-existing episode of Actinobacillosis which might obscure the interpretation of the infection experiment.

At four weeks-old, pigs were transported to the animal house and divided at random into five groups of eight pigs each, according to the same stratified randomisation procedure as used in Study 1.

On the day of arrival, all pigs were weighed and identified by a uniquely numbered eartag. The pigs were then allowed to acclimatize for four weeks.

Pigs were inoculated intranasally with 10^7 cfu of an *A. pleuropneumoniae* serotype 5a strain. Nine days after infection, half of the remaining pigs in each pen were euthanased and necropsied. The other pigs were necropsied at ten days post-infection. This was due to the time constraints of conducting this number of necropsies. Weighing of the pigs was performed three times: at the day of arrival (four weeks of age), at six weeks of age and at necropsy (ten weeks of age).

Sera were tested for neutralizing antibodies against ApxI and ApxII produced in recombinant *Escherichia coli* using a bioassay based on neutral red uptake by viable pulmonary alveolar macrophages (PAM) and titres of antibodies against ApxI were determined as described by Dom *et al.* (1994b).

Antibodies against ApxI and ApxII had been detected in 39 percent and 66 percent of the blood samples taken five weeks before infection. Therefore, blood samples were taken three weeks before the beginning of the study to differentiate between antibodies of active and passive immunity against *A. pleuropneumoniae.*

The important events in the study are summarised in the following schedule:

Schedule of events:

Day 1	Take rectal temperatures
Day 2	Take rectal temperature, measure water intake, weigh pigs, experimental infection of animals
Days 3-10	Clinical scores, rectal temperatures, water intake
Day 11	Euthanase and necropsy first half of surviving pigs
Day 12	Euthanase and necropsy remaining pigs

Results

Serology

Results are summarized in Table 9. At 36 days before infection, antibodies against ApxI and ApxII were detected in 39 percent and 66 percent of the animals, respectively. At 21 days before infection, these had declined to 25 percent and 35 percent against ApxI and ApxII, respectively. Since titres of antibodies and number of animals with antibodies was higher at 36 days before inoculation, those antibodies were likely to be of maternal origin.

Table 9: Results of serological testing for antibodies against Apx I and Apx II toxins at 36 and 21 days prior to inoculation for five groups of eight pigs prior to experimental infection with *A.pleuropneumoniae* serotype 5a

Group	Number of pigs with antibodies against ApxI and ApxII/total number of pigs (%) at			
	36 days before inoculation		21 days before inoculation	
	ApxI	ApxII	ApxI	ApxII
1	2/5 (40)	4/5 (80)	1/8 (13)	2/8 (25)
2	4/7 (57)	5/7 (71)	3/8 (38)	3/8 (38)
3	4/7 (57)	4/7 (57)	2/8 (25)	2/8 (25)
4	1/7 (14)	6/7 (86)	2/8 (25)	4/8 (50)
5	2/7 (29)	2/6 (33)	2/8 (25)	3/8 (38)
mean percentage	**39%**	**66%**	**25%**	**35%**

Feed Intake

The animals consumed all of the allocated 65 g feed per kilogram bodyweight each day. During the experimental study period, every pen therefore consumed 190 kg of grower food. Water intake data is summarised in Table 10.

Disease signs

Disease signs were not observed before inoculation. Pigs in all groups developed fever and disease signs shortly after inoculation. In addition, average rectal temperature and clinical demeanour scores for days six to ten days after inoculation were closely aligned with the average percent lung lesions found at necropsy.

Necropsy findings

Results are summarised in Table 11. It can be seen that total lung weight was lowest in group 4 . In groups 2 and 4, the number of pigs with lung lesions was lower than in other groups. The mean percentage of lung tissue with lesions was also lower in these groups.

Table 10: Daily water usage by five groups of eight pigs after experimental infection with *A. pleuropneumoniae* serotype 5a

Days after infection	Daily Water Usage (litres) per group				
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**
0	17.5	12.25	17	19	13.75
1	18	26.25	15.75	35	13.5
2	21.75	21.75	17.25	28	15.75
3	18	23	13.75	23.25	16.75
4	17.5	19.75	13.75	12.25*	15.75
5	17.75	22.5	15.75	26.75	16
6	18.25	21.5	12.75	- *	15.25
7	16	19.25	15.5	26.75	15.25
8	15	23	15	25.5	14.5
9	15	21.5	13.25	26	13
Mean	17.5	21.1	15	26.3**	15

N.B. * Due to a malfunction of the drinking water system, the barrel with drinking water ran empty on both occasions and so water uptake could not be measured.

** This mean was calculated without the water intake on Days 6 and 8.

Table 11: Necropsy findings and bacteriology results for five groups of eight pigs experimentally infected with *A. pleuropneumoniae* (APP) serotype 5a

Group	Total lungweight (kg)	Number of pigs/ total number with abcess-like nodules or pneumonia (%)	Mean percentage of lung tissue with lesions (%)	Total number with positive isolation of *APP*/ Number of pigs sampled for the following tissues: Lungs	Nostrils/tonsils
1	4.6	5/8 (63)	20	5/8 (63)	0/8 (0)
2	4.2	2/8 (25)	6	1/8 (13)	0/8 (0)
3	5.1	5/8 (63)	23	6/8 (75)	1/8 (13)
4	3.8	2/8 (25)	1	2/8 (25)	1/8 (13)
5	4.9	5/8 (63)	14	6/8 (75)	0/8 (0)

Bacteriology

Results are summarized in Table 11. From this table it can be concluded that *A. pleuropneumoniae* was isolated from lungs of pigs in all groups. The number of lungs from which *A. pleuropneumoniae* was isolated was lowest in groups 2 and 4.

Bodyweight

Results are summarized in Table 12.

Table 12: Summarized clinical, growth and pathological data for five groups of eight pigs experimentally infected with *A. pleuropneumoniae* serotype 5a

	Days after infection	Group				
		1	2	3	4	5
Deaths		1	0	1	0	0
Mean rectal Temperature (°C)	1-4	40.1	40.1	39.9	39.6	39.9
	5-9	39.8	39.4	39.9	39.2	39.8
Mean clinical Score	1-4	0.82	0.44	1.09	0.67	0.91
	5-9	0.46	0.25	0.46	0.08	0.50
Lung Weight (g)	9-10	577	530	633	478	613
(% of bodyweight)		2.92	2.70	3.36	2.37	2.85
Percent Lung Lesions	9-10	20.0	5.6	23.1	1.3	14.4
Bodyweight (kg)	-18	9.5	9.9	9.2	9.3	9.0
	9	20.1	19.9	19.6	20.1	21.3
Weight Gain (kg) (27 days)		10.6	10.0	10.4	10.8	12.3

In some animals, equally distributed between different groups, antibodies were detected at 21 days before inoculation. Since antibody titres and seroprevalence were higher 36 days before inoculation, those antibodies were more likely of maternal origin. This situation reflects the field situation since the prevalence of *A. pleuropneumoniae* is very high in Belgium (Dom *et al.* 1994b), where this study was conducted. All animals, including those with neutralising antibodies, showed febrile or clinical responses after infection.

One pig developed peracute pleuropneumonia and died after experimental infection. All other animals developed acute disease. *A. pleuropneumoniae* was re-isolated from the lungs from at least one animal in every group. This indicates that the experimental infection model worked.

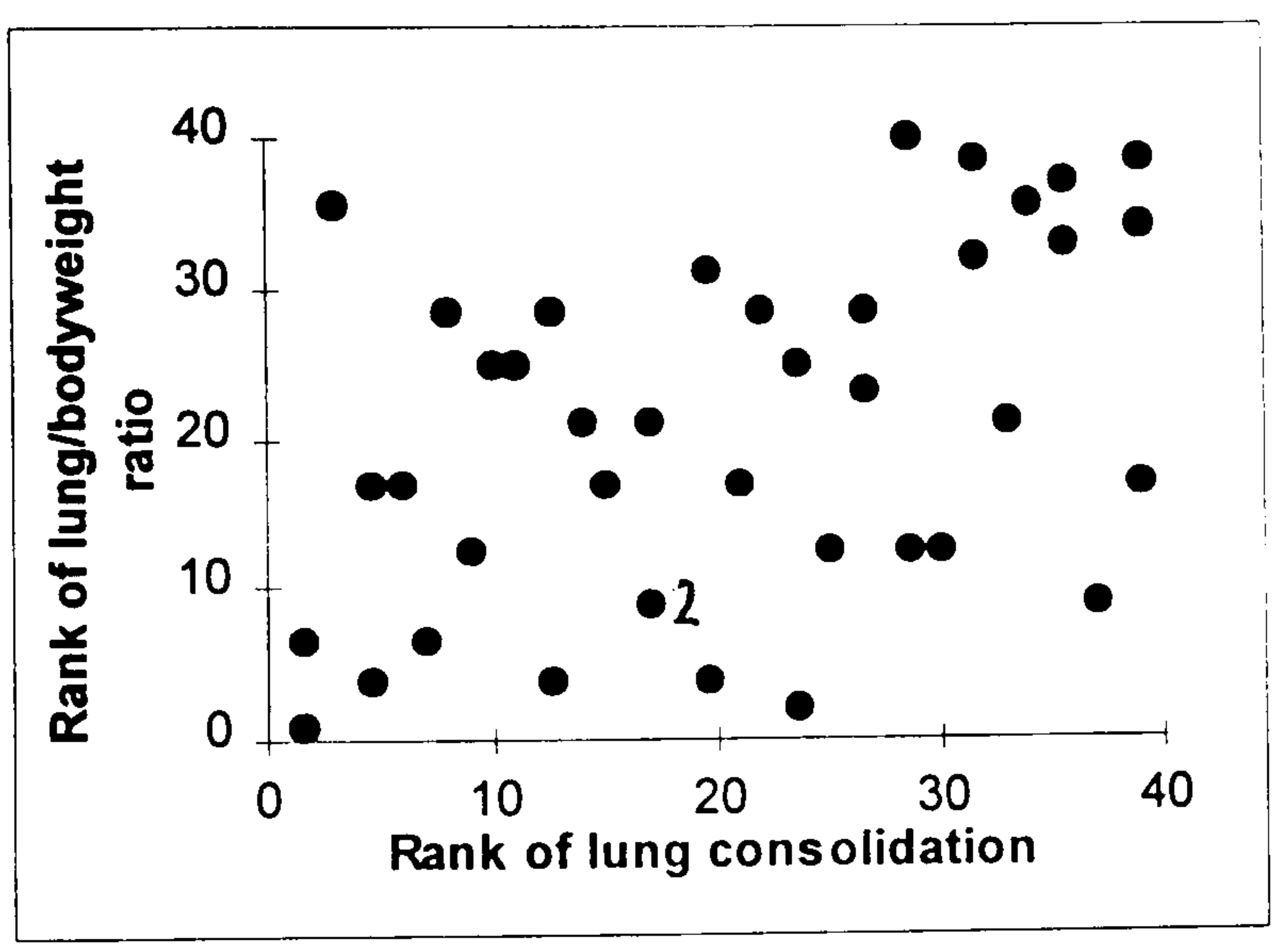
Table 13: Clinical and pathological data and ranked data from pigs experimentally infected with *A. pleuropneumoniae* serotype 5a

PBE9501 Clinical score	Rectal Temp. (°C)	Resp. score	Lung consoln. (%)	Lung/ Bwt ratio	Bacteriol. reisolation	RANKS Clinical score	RANKS Rectal Temp. (°C)	RANKS Resp. score	RANKS Lung consoln. (%)	RANKS Lung/ Bwt ratio	RANKS Bacteriol. reisolation
0	39.9	0	20	2	1	19	27.5	16.5	12.5	4	30.5
0	39.9	0	95	3.6	1	19	27.5	16.5	35.5	33	30.5
0	40.9	0	60	5.2	1	19	37	16.5	28.5	40	30.5
0	40.3	1	35	2.3	1	19	33.5	35.5	17	9	30.5
0	40	0	85	2.7	1	19	30	16.5	33	21	30.5
0	39.6	0	2.5	2.6	0	19	21.5	16.5	6	17	10.5
0	40.1	1	0	2.2	0	19	31	35.5	1.5	6.5	10.5
3	41	3	70	2.4	0	39.5	38.5	39.5	30	12.5	10.5
0	39.1	0	50	1.8	0	19	3	16.5	23.5	2	10.5
0	39.2	0	50	2.9	0	19	4.5	16.5	23.5	25	10.5
0	38.9	0	25	2.7	0	19	1.5	16.5	14	21	10.5
0	39.2	0	5	3	0	19	4.5	16.5	8	28.5	10.5
0	39.4	0	90	4	1	19	9	16.5	34	35.5	30.5
0	39.4	0	55	3	0	19	9	16.5	26.5	28.5	10.5
0	38.9	0	0	1.4	0	19	1.5	16.5	1.5	1	10.5
0	39.7	0	40	3.4	0	19	25	16.5	19.5	31	10.5
0	39.6	0	1	4	1	19	21.5	16.5	3	35.5	30.5
0	39.9	0	20	3	1	19	27.5	16.5	12.5	28.5	30.5
0	39.9	0	45	3	0	19	27.5	16.5	22	28.5	10.5
0	39.4	0	100	4.3	1	19	9	16.5	39	38.5	30.5
3	41	3	100	2.6	0	39.5	38.5	39.5	39	17	10.5
0	40.3	0	18	2.9	1	19	33.5	16.5	11	25	30.5
0	40.4	1	95	4.2	1	19	35.5	35.5	35.5	37	30.5
0	39.4	1	55	2.8	1	19	9	35.5	26.5	23	30.5
0	39.6	0	30	2.6	0	19	21.5	16.5	15	17	10.5
0	39.4	0	2	2.6	0	19	9	16.5	4.5	17	10.5
0	39.4	0	35	2.7	0	19	9	16.5	17	21	10.5
0	39.5	0	4	2.2	0	19	15.5	16.5	7	6.5	10.5
0	39.6	0	35	2.3	0	19	21.5	16.5	17	9	10.5
0	39.5	0	40	2	0	19	15.5	16.5	19.5	4	10.5
0	39.5	0	2	2	1	19	15.5	16.5	4.5	4	30.5
0	41.3	0	97	2.3	1	19	40	16.5	37	9	30.5
0	40.2	1	42	2.6	1	19	32	35.5	21	17	30.5
0	39.5	0	12	2.9	1	19	15.5	16.5	10	25	30.5
0	39.5	0	100	3.7	1	19	15.5	16.5	39	34	30.5
0	40.4	0	10	2.4	0	19	35.5	16.5	9	12.5	10.5
0	39.4	1	75	4.3	1	19	9	35.5	31.5	38.5	30.5
0	39.6	0	60	2.4	1	19	21.5	16.5	28.5	12.5	30.5
0	39.5	0	54	2.4	1	19	15.5	16.5	25	12.5	30.5
1	39.6	0	75	3.5	0	38	21.5	16.5	31.5	32	10.5

N.B. consoln. = consolidation; Bwt = Bodyweight

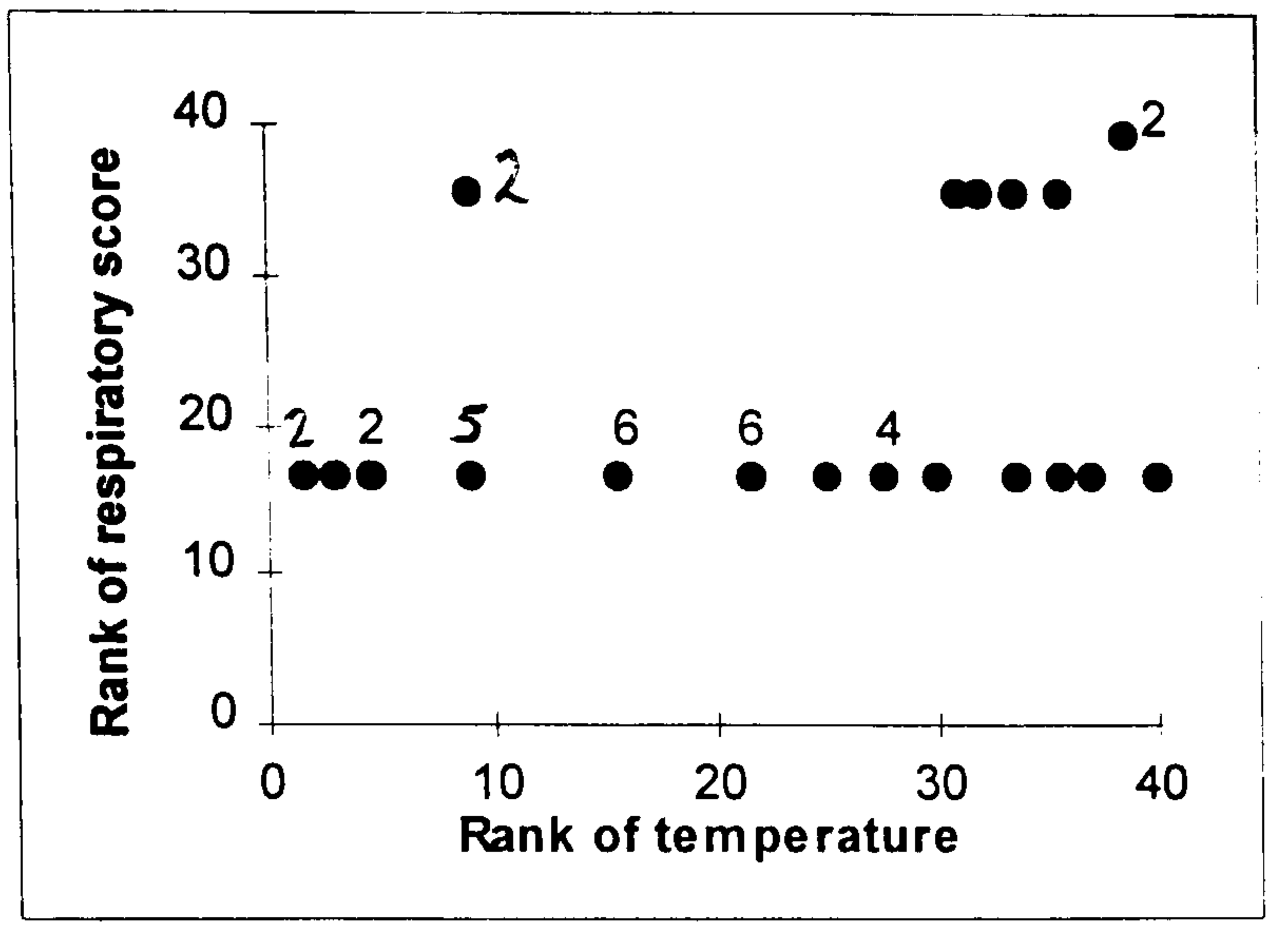
The correlation between lung consolidation and lung/bodyweight ratio was highly significant (p=0.005), yet, Figure 4 also illustrates that there is much individual variability, and that the correlation should only be assumed when analysing group data. Three other correlations were significant at the 5 percent level: rectal temperature was positively correlated with respiratory score (Figure 5 and Table 21a), and bacteriological isolation (Table 21a) was positively correlated with both lung consolidation and lung/bodyweight ratio (Figures 4 and 6). Correlation coefficients involving clinical score were not calculated as there were only three animals in this study with non-zero scores.

Figure 4: Graphical illustration of the relationship between ranked data for lung/bodyweight ratio and lung consolidation for 40 pigs experimentally infected with *A. pleuropneumoniae* serotype 5a



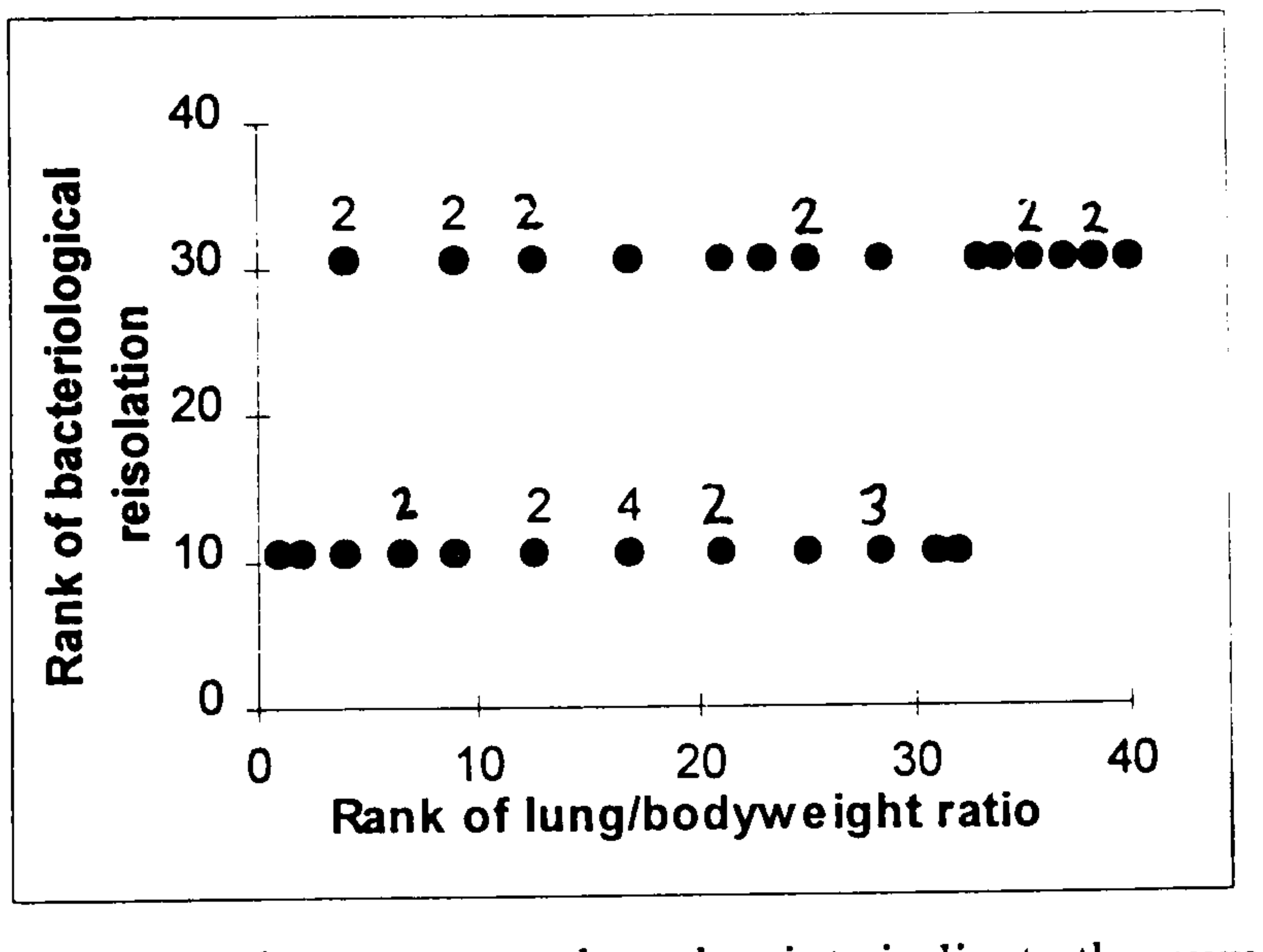
N.B. Numbers next to plotted points indicate the number of identical values.

Figure 5: Graphical illustration of the relationship between ranked data for respiratory score and rectal temperature for 40 pigs experimentally infected with *A. pleuropneumoniae* serotype 5a



N.B. Numbers next to plotted points indicate the number of identical values.

Figure 6: Graphical representation of the relationship between ranked data for bacteriological reisolation and lung/ bodyweight ratio for 40 pigs experimentally infected with *A. pleuropneumoniae* serotype 5a



N.B. Numbers next to plotted points indicate the number of identical values.

Study 3-Pigs (NE9501)

Overview

Fifty specific-pathogen-free (SPF) pigs were used in this study. The pigs were selected and weighed when they were approximately eight-weeks-old. Groups of ten pigs were split between two replicates.

In the early morning of Day 9, every pig was endobronchially inoculated with 10^4 *A. pleuropneumoniae* serotype 9. First clinical signs were observed 6-8 hours after challenge and all pigs were 'very dull' (demeanour score 3) 12 hours after challenge. All pigs were dyspnoeic with abdominal breathing, and an increased respiratory rate. All but one pig was pyrexic. Marked coughing was observed in all experimental groups. Vomitus was found on the floor of all pens. Twenty-four hours after challenge, most of the pigs were 'severely diseased' (demeanour score 4). It was decided to euthanase the 'severely diseased' pigs for welfare reasons. Remaining pigs were euthanased the next day. At necropsy, lungs of all pigs had typical lesions with haemorrhages, necrosis and fibrin. *A. pleuropneumoniae* serotype 9 was isolated from all swab specimens taken from the lung lesions from all pigs. Additional to the data examined in the previous two studies (i.e. clinical demeanour score: rectal temperature; respiratory score; percent lung consolidation; lung/bodyweight ratio), cough score, weight gain, tracheo-bronchial, thoracic and sternal lymph node enlargement were also examined as potential indicators of disease severity.

Materials and methods

A. pleuropneumoniae strain 13261, serotype 9 was used. The strain was originally isolated from a lung of a pig from a recent field case of pleuropneumonia. A laboratory strain was passed through an SPF-pig and the first passage of strain was stored in Eagle's Minimal Essential Medium supplemented with Earle's salts (EMEM; ICN-Flow lab.) at -70°C. One vial with a suspension of *A. pleuropneumoniae* 13261 was thawed from - 70°C . A heart infusion agar plate supplemented with 5% defibrinated sheep blood and 0.2% nicotinamide adenine dinucleotide (HIS+V plate) was inoculated with a loopful of this suspension. The plate was incubated overnight at 37°C and 5% CO_2. The next morning 5 haemolytic colonies were suspended in 1 ml EMEM. This suspension was heavily inoculated on 2 HIS+V plates by use of a cotton swab. These plates were incubated for 8 hours at 37°C at 5% CO_2. Then, bacterial growth was washed off with 5 ml EMEM per plate. Washings were pooled. One sample was used for a Gram-stain and confirmed that the pool consisted only of small Gram negative rods. Another sample was agglutinated with an antiserum against *A. pleuropneumoniae* serotype 9 and confirmed that it was serotype 9. Duplicates of ten-fold serial dilutions of another sample were plated on HIS+V to determine the number of Colony Forming Units (CFU). The remainder of the pooled suspension was stored at 4°C. The next morning, the number of CFU was counted and the inoculum was prepared by diluting the bacterial suspension in EMEM to a concentration of approximately 10^3 bacteria per ml. This dose was expected to give 100% morbidity and approximately 30% mortality in the untreated control group based upon previous investigators use of a similar infection protocol (Pijpers *et al.* 1990a, 1990b, Mengelers *et al.* 1991). A 1000:1 dilution of this inoculum was plated on HIS+V to confirm the number of CFU. The inoculum was divided into five bottles, one for each MPB. The inoculum was transported on ice to the MPBs and what was left after inoculation was returned to the laboratory. Again a 1000:1 dilution of this inoculum was plated on HIS+V to check the number of viable bacteria.

Inoculation of the pigs

Pigs were inoculated early in the morning of Day 9 of the experiment.

Pigs were anaesthetised by an open inhalation system. A cone-shaped face mask was fixed upon the pig's nose and a gas mixture of 48% oxygen, 48% nitrous oxide and 4% halothane was administered for 3-4 minutes. The gas flow was fixed at 2000 ml per minute for both oxygen as well as nitrous oxide. Then, the mask was removed and the pig was held with the head in a vertical position. The pig's mouth was held open with a gag. A laryngoscope was introduced in the mouth and used to press down the epiglottis. A sterile stiff catheter (Cordis Europe, Etten Leur, The Netherlands) was introduced into the trachea until some resistance was felt. Ten millilitres of the inoculum was slowly injected via the catheter deep into the lung with a 10 ml disposable syringe (Terumo Europe, Leuven Belgium). The catheter was then quickly withdrawn.

Bacteriological examination of lung samples after necropsy

Swab samples from lung lesions were cultured on HIS + V plates. Plates were incubated overnight at 37^0C and 5% CO_2. Two to three haemolytic colonies were tested in a slide agglutination assay with antiserum directed against *A. pleuropneumoniae* serotype 9 to confirm the causative organism.

Results

Challenge dose

The number of bacteria in the challenge dose was counted before and after inoculation. Bacterial counts varied from $1.2x10^3$ to $1.8x10^3$ per ml.

Water intake

Daily water intakes before challenge were similar for all groups, after inoculation water intake for all groups dropped to between 23 percent and 38 percent of the pre-inoculation intakes, indicating the effect that *A. pleuropneumoniae* may have on water intake (Table 14). There was no anaesthetised, non-inoculated control group, so it cannot be judged what effect the anaesthesia may have had in depressing water intake.

Table 14: Water intake in litres per day for groups of ten pigs up to and including the day of endobronchial inoculation with *A. pleuropneumoniae serotype* 9 (Day 9)

	Group				
Day	1	2	3	4	5
5	16.6	16.3	11.6	13.9	14.7
6	30.2	25.0	20.9	25.2	25.7
7	15.1	13.5	10.7	12.8	12.5
7	19.6	19.7	10.4	14.5	12.4
8	37.2	32.0	28.5	28.3	37.4
9	14.0	9.0	7.2	5.5	8.7

Feed uptake and weight gains

Feed uptake of the groups were similar in the period before challenge, as were weight gains during this period (Table 15). After challenge weight gains were reduced and many pigs lost weight even in the short period between inoculation and euthanasia 24 to 48 hours later (Table 15). The mean daily weight gain is given in Table 16 for each group.

Table 15: Feed uptake and weight gains (kilograms) for groups of ten pigs experimentally infected with *A. pleuropneumoniae* serotype 9

Group	feed uptake day 0-end (kg)	mean weight gain day 0-8 (kg)	mean weight gain after challenge (a.c.) of pigs euthanased	
			one day a.c.	two days a.c.
1	107.5	4.5 ± 1.5	0.1 ± 1.6	
2	99.6	4.7 ± 0.9	-0.4 ± 0.9	-0.6 ± 1.0
3	96.0	4.2 ± 0.9	-0.6 ± 0.7	-0.9 ± 0.6
4	93.8	4.8 ± 2.1	-0.7 ± 0.8	-0.1 ± 1.8
5	110.8	5.4 ± 1.5	0.5 ± 1.4	0.4 ± 2.1

Table 16: Mean daily weight gains of the pigs (kilograms) for groups of ten pigs experimentally infected with *A. pleuropneumoniae* serotype 9

	mean weight gain per day before challenge (kg)	mean weight gain (kg) per day after challenge of pigs euthanased at	
Group		Day 10	Day 11
1	0.57 ± 0.19	0.07 ± 0.8	-
2	0.59 ± 0.11	-0.21 ± 0.43	-0.16 ± 0.40
3	0.52 ± 0.12	-0.37 ± 0.33	-0.43*
4	0.59 ± 0.26	-0.33 ± 0.42	-0.04 ± 0.60
5	0.67 ± 0.19	0.25*	0.04 ± 0.58

* too few data to calculate a standard deviation.

Clinical observations

First clinical signs of respiratory disease were observed approximately 6-8 hours after challenge when all pigs had demeanour scores 2 and 3. All had dyspnoea with abdominal breathing, and an increased respiratory rate. Marked coughing was observed in all experimental groups. Vomitus was found on the floor of all pens and some pigs were seen vomiting. Clinical scores were high in all groups and all pigs had typical lesions at necropsy from which *A. pleuropneumoniae* was isolated in pure culture.

Necropsy findings

Except for pig 2298, all pigs that were euthanased one day after challenge had large quantities of exudate with fibrin in the pleural cavity. Most pigs that were euthanased on the second day after challenge had severe pleuritis with much fibrin in the pleural cavity. Lungs of all pigs had characteristic lesions with haemorrhages, necrosis and fibrin.

In general, thoracic lymph nodes of all but one pig (2295) were enlarged (lymph node score 1-3). Peritoneal cavities, spleens, and hearts of pigs in all groups appeared macroscopically normal or slightly enlarged. The kidneys and liver of many pigs of all groups were swollen.

A summary of the clinical and pathological data is given in Table 17, and the ranked data used to graphically correlate it in Table 18.

Table 17: Summarised clinical data for 50 pigs artificially infected with *A. pleuropneumoniae* serotype 9

PNE9501 Pig no.	Clinical score	Rectal Temp. (°C)	Resp. score	Lung consoln. (%)	Lung/ Bwt ratio	Cough Score	Weight gain (%)	Tracheo bronchial lymph node score	Thoracic lymph node score	Sternal aortic lymph node score
2281	3	41.1	2	40	2.10	0	47.73	2	2	1
2282	3	40.1	3	10	1.25	0	40.00	1	1	1
2283	3	42	2	40	1.96	2	37.89	3	2	1
2284	3	40.7	2	40	1.89	2	46.32	2	2	1
2285	3	40.3	2	50	1.87	2	40.53	3	2	1
2286	4	40.6	2	30	1.56	2	43.24	2	2	1
2287	3	40.9	1	25	1.14	2	43.92	3	2	1
2288	3	41.2	3	40	2.64	2	38.06	2	1	2
2289	3	40.7	2	40	1.71	0	48.39	2	2	1
2291	3	40.7	3	25	1.09	2	37.83	2	2	1
2292	3	41.3	2	40	1.62	0	36.52	3	2	1
2293	3	40.4	3	.40	2.07	1	20.87	2	2	1
2294	3	40.8	2	60	2.00	0	43.00	2	2	1
2295	3	40.2	2	2.5	0.91	0	56.50	0	0	0
2296	3	41.9	2	40	1.50	2	36.32	3	2	1
2297	3	40.4	2	40	1.69	0	39.47	2	2	1
2298	2	40	2	5	0.96	0	57.14	1	1	0
2299	3	40.7	3	15	1.10	2	61.71	3	2	1
2301	3	40.2	2	40	1.69	0	43.48	2	2	1
2302	3	41.5	2	40	1.46	2	37.27	2	1	1
2303	3	39.9	2	60	3.08	2	30.48	3	2	1
2304	3	40.7	3	60	2.68	0	50.50	2	1	1
2305	3	41.4	2	30	1.29	0	44.62	2	1	1
2306	3	40.7	2	40	1.79	2	54.36	2	2	1
2307	3	41.4	2	40	1.98	2	42.63	2	2	1
2309	3	41	2	25	1.85	2	44.78	2	2	1
2310	3	41	2	30	1.59	0	18.70	2	2	1
2311	3	40.2	2	30	1.07	0	42.79	2	1	1
2312	3	42.1	2	30	1.82	0	36.50	2	2	1
2313	4	40.8	3	30	1.62	2	36.00	3	2	1
2314	3	40.9	1	40	1.69	2	50.26	2	2	1
2315	3	39.8	2	25	1.39	0	45.26	3	2	1
2316	3	40.9	2	25	1.15	2	43.24	2	2	1
2317	4	41.4	2	50	1.86	0	46.67	2	2	1
2318	3	41.4	2	30	1.71	0	40.00	2	1	1
2319	3	40.8	2	50	1.59	2	48.44	2	1	2
2320	3	40.7	2	33	1.35	1	42.73	2	0	2
2321	3	40.7	2	40	1.23	2	36.82	2	2	1
2322	2	41.3	1	40	1.62	0	31.63	2	2	1
2323	3	40.3	3	15	0.95	0	42.38	2	2	1
2324	3	40.4	2	50	2.88	2	33.33	2	2	1
2325	3	39.6	2	8	0.87	0	55.14	2	1	0
2326	3	40.5	2	40	1.89	2	38.38	3	3	1
2327	3	40.3	2	40	1.74	0	50.00	2	2	1
2328	3	39.6	2	20	1.34	0	26.05	3	2	1
2329	4	40.4	2	30	1.57	0	36.19	3	3	1
2330	4	39.7	2	10	1.31	0	33.33	3	2	2
2331	4	41.5	3	70	2.96	2	39.05	2	1	1

2332	3	41.7	2	40	1.85	1	32.22	2	2	1
2333	3	40.9	2	40	1.59	1	31.58	2	2	1

N.B. consoln. = consolidation; Bwt = Bodyweight

Table 18: Ranked data for 50 pigs artificially infected with *A. pleuropneumoniae* serotype 9

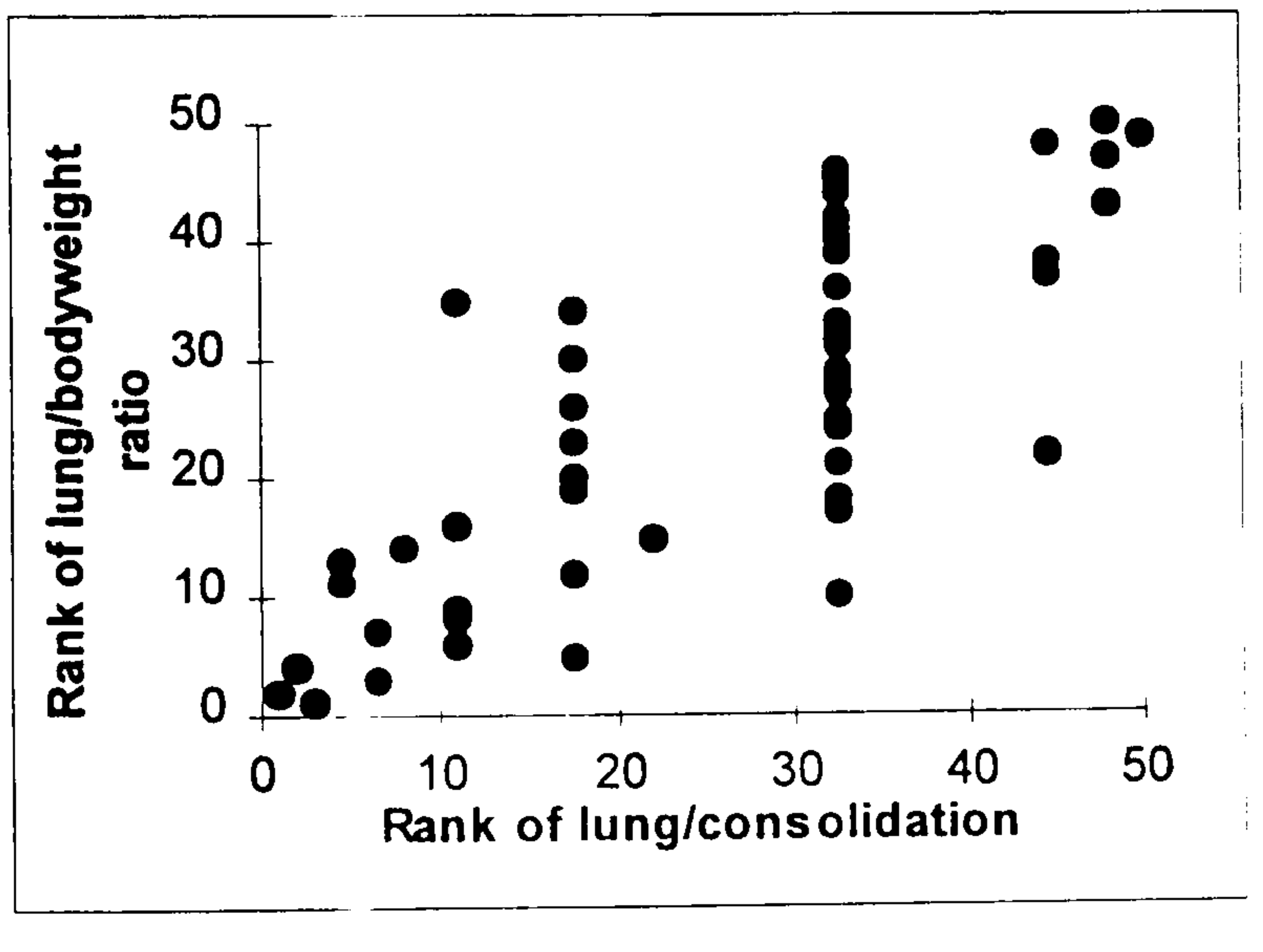
PNE9501 Pig no.	RANKS Clinical score	RANKS Rectal Temp. (°C)	RANKS Resp. score	RANKS Lung consoln. (%)	RANKS Lung/ Bwt ratio	RANKS Cough	RANKS Weight gain (%)	RANKS Tracheo bronchial lymph node score	RANKS Thoracic lymph node score	RANKS Sternal aortic lymph node score
2281	23.5	37	22.5	32.5	45	12.5	40	20.5	31	25
2282	23.5	7	46	4.5	11	12.5	23.5	2.5	8	25
2283	23.5	49	22.5	32.5	41	39.5	18	44	31	25
2284	23.5	23.5	22.5	32.5	40	39.5	38	20.5	31	25
2285	23.5	12	22.5	44.5	38	39.5	25	44	31	25
2286	47.5	19	22.5	17.5	19	39.5	31.5	20.5	31	25
2287	23.5	32.5	2	11	8	39.5	34	44	31	25
2288	23.5	38	46	32.5	46	39.5	19	20.5	8	48.5
2289	23.5	23.5	22.5	32.5	31	12.5	41	20.5	31	25
2291	23.5	23.5	46	11	6	39.5	17	20.5	31	25
2292	23.5	39.5	22.5	32.5	25	12.5	14	44	31	25
2293	23.5	15.5	46	32.5	44	26.5	2	20.5	31	25
2294	23.5	29	22.5	48	43	12.5	30	20.5	31	25
2295	23.5	9	22.5	1	2	12.5	48	1	1.5	2
2296	23.5	48	22.5	32.5	18	39.5	12	44	31	25
2297	23.5	15.5	22.5	32.5	29	12.5	22	20.5	31	25
2298	1.5	6	22.5	2	4	12.5	49	2.5	8	2
2299	23.5	23.5	46	6.5	7	39.5	50	44	31	25
2301	23.5	9	22.5	32.5	27	12.5	33	20.5	31	25
2302	23.5	45.5	22.5	32.5	17	39.5	16	20.5	8	25
2303	23.5	5	22.5	48	50	39.5	4	44	31	25
2304	23.5	23.5	46	48	47	12.5	45	20.5	8	25
2305	23.5	42.5	22.5	17.5	12	12.5	35	20.5	8	25
2306	23.5	23.5	22.5	32.5	33	39.5	46	20.5	31	25
2307	23.5	42.5	22.5	32.5	42	39.5	27	20.5	31	25
2309	23.5	35.5	22.5	11	35	39.5	36	20.5	31	25
2310	23.5	35.5	22.5	17.5	23	12.5	1	20.5	31	25
2311	23.5	9	22.5	17.5	5	12.5	29	20.5	8	25
2312	23.5	50	22.5	17.5	34	12.5	13	20.5	31	25
2313	47.5	29	46	17.5	26	39.5	10	44	31	25
2314	23.5	32.5	2	32.5	28	39.5	44	20.5	31	25
2315	23.5	4	22.5	11	16	12.5	37	44	31	25
2316	23.5	32.5	22.5	11	9	39.5	31.5	20.5	31	25
2317	47.5	42.5	22.5	44.5	37	12.5	39	20.5	31	25
2318	23.5	42.5	22.5	17.5	30	12.5	23.5	20.5	8	25
2319	23.5	29	22.5	44.5	22	39.5	42	20.5	8	48.5
2320	23.5	23.5	22.5	22	15	26.5	28	20.5	1.5	48.5
2321	23.5	23.5	22.5	32.5	10	39.5	15	20.5	31	25
2322	1.5	39.5	2	32.5	24	12.5	6	20.5	31	25
2323	23.5	12	46	6.5	3	12.5	26	20.5	31	25

2324	23.5	15.5	22.5	44.5	48	39.5	8.5	20.5	31	25
2325	23.5	1.5	22.5	3	1	12.5	47	20.5	8	2
2326	23.5	18	22.5	32.5	39	39.5	20	44	49.5	25
2327	23.5	12	22.5	32.5	32	12.5	43	20.5	31	25
2328	23.5	1.5	22.5	8	14	12.5	3	44	31	25
2329	47.5	15.5	22.5	17.5	20	12.5	11	44	49.5	25
2330	47.5	3	22.5	4.5	13	12.5	8.5	44	31	48.5
2331	47.5	45.5	46	50	49	39.5	21	20.5	8	25
2332	23.5	47	22.5	32.5	36	26.5	7	20.5	31	25
2333	23.5	32.5	22.5	32.5	21	26.5	5	20.5	31	25

N.B. consoln. = consolidation; Bwt = Bodyweight

The strongest correlation was between lung consolidation and lung/bodyweight ratio (Spearman's coefficient, 0.79; $p<0.001$) (Figure 7 and Table 21b). Temperature showed statistically significant correlations with both of these parameters, but these relationships were not very strong.

Figure 7: Graphical illustration of the relationship between ranked data for lung/ bodyweight ratio and lung consolidation for 50 pigs artificially infected with *A. pleuropneumoniae* serotype 9



The scores for tracheo-bronchial and thoracic lymph nodes showed a highly significant positive correlation ($p<0.001$) (Table 19 and Table 21b). Weight gain showed statistically significant negative correlations with both of these parameters even over the short interval between infection and euthanasia due to the near total inappetance of pigs (Table 21b). The only other correlation significant at the 5 percent level was between clinical score and tracheo-bronchial lymph nodes (Tables 20 and 21b).

Table 19: Two way table showing relationship between increase in lymph node size for tracheo-bronchial and thoracic lymph nodes for 50 pigs artificially infected with *A. pleuropneumoniae* serotype 9

No. of animals	Tracheo bronchial lymph nodes 0 or 1	2	3
Thoracic lymph nodes			
0	1	1	0
1	2	9	0
2	0	24	11
3	0	0	2

Table 20: Two way table showing numbers of pigs infected with *A. pleuropneumoniae* with hyperplasia of the tracheo-bronchial lymph nodes at increasing clinical scores for 50 pigs artificially infected with *A. pleuropneumonia* serotype 9

No. of animals	Clinical score 2	3	4
Tracheo bronchial lymph nodes			
0 or 1	1	2	0
2	1	30	3
3	0	10	3

Discussion (Study 3)

A model in which SPF pigs were deeply endobronchially inoculated with *A. pleuropneumoniae* serotype 9 at a challenge dose of approximately 10^4 bacteria per pig was chosen because this dose gave 100 percent morbidity.

After challenge, all pigs became very dull (score 3) within 12 hours. Water intake and feed intake was markedly reduced after challenge. The animal model used is clearly a severe model and this limited its usefulness. The natural infection route of *A. pleuropneumoniae* in pigs is aerogenous. In this model, bacteria were inoculated directly deep into the bronchi and thus the defence mechanisms of the upper respiratory tract were circumvented. The advantage of endobronchial inoculation model is that it is very reproducible and gives 100 percent morbidity in control pigs. Furthermore, the number of bacteria that reach the lungs is known. The model has been used extensively elsewhere to prove protection of vaccination (Kamp *et al.* 1992) and to study the effects of in-feed medication with tetracyclines and combinations of sulfonamides and trimethroprim (Pijpers *et al.* 1990a, 1990b, Mengelers *et al.* 1991). In these studies, the challenge doses were 10^4 CFU/pig, but conventional pigs instead of SPF-pigs were used. Conventional pigs appear to be less susceptible than SPF-pigs and higher challenge doses are needed for similar severity of disease to be obtained. Quantification of the inoculum titre after inoculation showed that pigs were inoculated with approximately 10^3 CFU per pig instead of the intended 10^4 CFU/pig which had been used previously in this model. Nevertheless, the disease severity was higher than in previous studies. This may be partly due to the fact that SPF pigs were used, and they may be more susceptible to infection.

Statistical analysis and discussion of pig study results

A number of variables from three different studies were available for analysis. In the first study, PUK9501, the variables were:

Clinical (demeanour) score
Temperature
Respiratory score
Percent lung consolidation
Lung/bodyweight ratio (%)

There were 58 animals with data available from this study, but one animal did not have data recorded for the last two of the above variables. Hence, the pairwise correlations were calculated from 57 or 58 observations, according to the data available.

In the second study, PBE9501, the same variables as listed above were available, and in addition bacteriological reisolation (0=no, 1=yes) was recorded. There were 40 animals in this study. However, the clinical score was zero for all but three of these animals, and so this variable has not been included in the correlation analysis for PBE9501.

In the third study, PNE9501, the same variables as in PUK9501 were recorded, as well as a number of additional variables:

Cough score
Weight gain (%)
Tracheo-bronchial lymph node score
Thoracic lymph node score
Sternal aortic lymph node score

There were 50 animals in this study. One lung consolidation value was recorded as '<5' and this has been set to 2.5 percent so that this observation could be used in the analysis.

The results presented consist of correlation coefficients and p-values for each pair of variables. In each case, Spearman's correlation coefficient was calculated, i.e. given n pairs of observations (x_i , y_i) (i=1,2,...,n) for two variables x and y the coefficient is:

$$\theta = \frac{\sum_{i=1}^{n}(r_i - \bar{r})(s_i - \bar{s})}{\sqrt{\sum_{i=1}^{n}(r_i - \bar{r})^2 \sum_{i=1}^{n}(s_i - \bar{s})^2}}$$

where r_i denotes the rank of x_i, s_i denotes the rank of y_i, and $\bar{r}$ and $\bar{s}$ denote the mean values of the ranks of x and y respectively. The p-value was obtained by calculating the probability of obtaining a correlation at least as big as the one observed for the given number of observations, under the hypothesis of there being no relationship between the two variables. This was done automatically using the CORR procedure in the SAS for Windows (Version 6.12) package. In the tables of results, correlations significant at the 5 percent level have been highlighted in bold (Tables 21a and 21b).

In study PUK9501, two highly significant correlations were noted, between clinical score and respiratory score, and between lung consolidation and lung/ bodyweight ratio ($p<0.001$). It should be noted that for half the animals in this study, both the clinical score and the respiratory score were zero. However, a chi-square analysis of the two-way table of clinical score versus respiratory score, with both scores recorded as zero or non-zero, also provided highly significant evidence of an association between these two variables ($p=0.001$). None of the other pairwise correlations in this study were significant at the 5 percent level.

In study PBE9501, the correlation between lung consolidation and lung/ bodyweight ratio was also highly significant, although not as strong as in the first study. Three other correlations were significant at the 5 percent level: temperature was positively correlated with respiratory score, and bacteriological isolation was positively correlated with both lung consolidation and lung/ bodyweight ratio. However, these positive correlations were not very strong. Correlations involving clinical score were not calculated as there were only three animals in this study with non-zero scores.

In study PNE9501, the strongest correlation was again between lung consolidation and lung/ bodyweight ratio. Temperature showed statistically significant correlations with both of these parameters, but these relationships were not very strong.

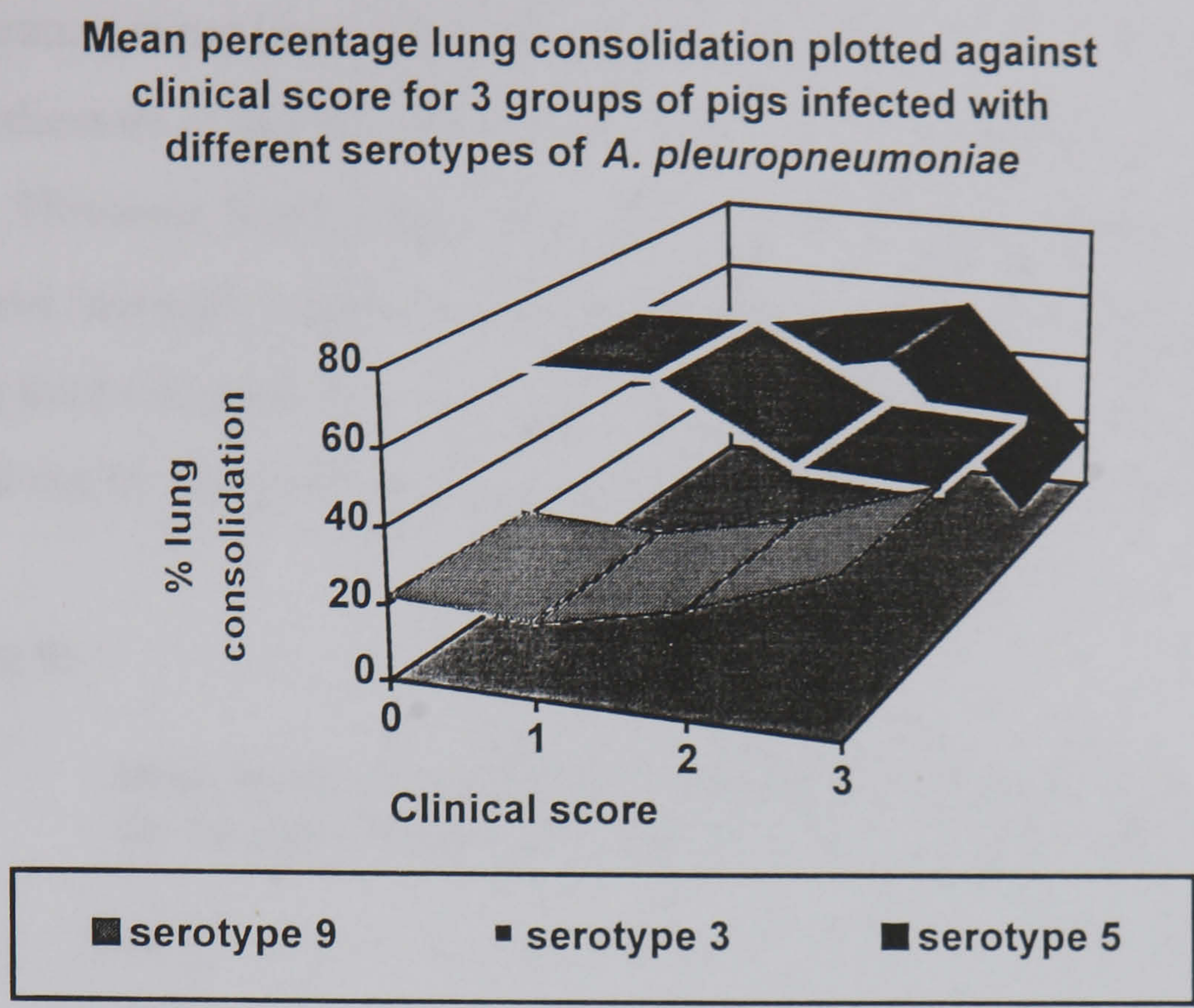
The scores for tracheo-bronchial and thoracic lymph nodes showed a highly significant positive correlation ($p<0.001$). Weight gain showed statistically significant negative correlations with both of these parameters, but these relationships were not strong. The only other correlation significant at the 5 percent level was between clinical score and tracheo-bronchial lymph nodes.

Care should be taken in the interpretation of statistical significance, particularly in the PNE9501 study, due to the number of tests being performed.

Looking at the three studies together, the positive correlation between lung consolidation and lung/ bodyweight ratio was consistent across the three studies, but there were many differences in terms of the other parameters. On pooling the data there were significant differences between the three studies in the relationships between the variables. Hence, calculating correlation coefficients for the combined data is not appropriate, and so these additional results have not been presented. These studies suggest that for *A. pleuropneumoniae* infections in pigs, clinical demeanour and respiratory scores do not give a reliable indication of the pathology occurring in the animal.

Figure 8 illustrates the different relationships observed with each serotype of *A. pleuropneumoniae* between the clinical condition of the pig and the gross pathology observed.

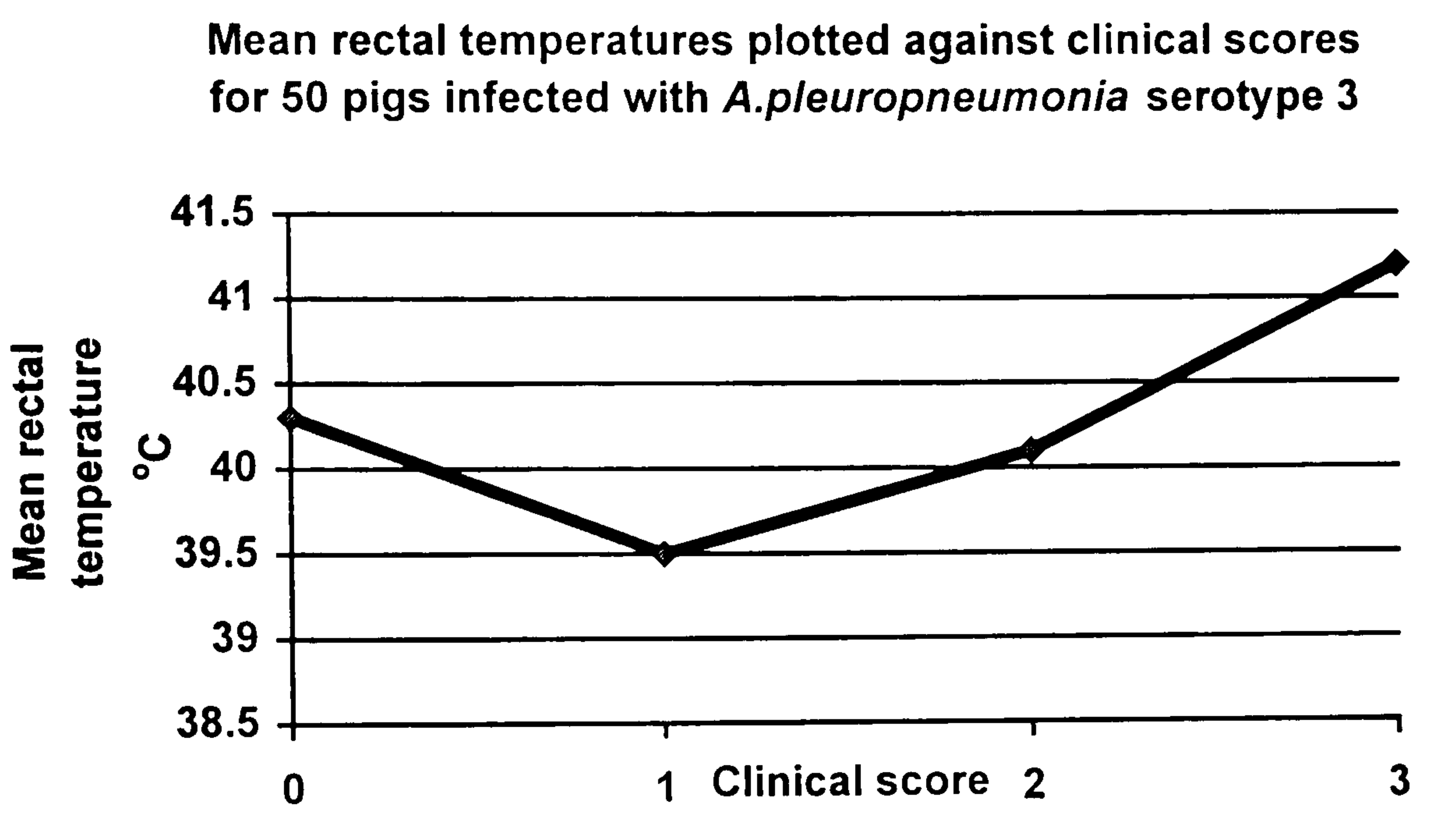
Figure 8:



Part of the reason for the different relationship between clinical and pathological measures is likely to be due to a difference in the virulence of different serotypes of *A. pleuropneumoniae*, and the release of different amounts of the RTX toxins. The deleterious effects of these proteins secreted by *A. pleuropneumoniae* have been demonstrated to be toxic to porcine erythrocytes, endothelial cells, macrophages and neutrophils (Udeze *et al.* 1987; Dom *et al.* 1994c). The differences in relationship in Figure 8 may also be due to the fact that pigs with per-acute signs were euthanased before the gross manifestation of the pathology were evident. Pigs with milder clinical signs survived until the end of the study and abscess formation in the lungs therefore developed further over the subsequent 14 days. In Study 3 (PNE9501), all pigs were necropsied at a similar interval after infection which possibly accounts for the more linear relationship between clinical and pathological signs seen with serotype 9 data in Figure 1. In the other two studies, some severely affected pigs were necropsied as little as one or two days after infection, while the rest were necropsied as late as the end of the study, 14 days later.

Figure 9 shows that the relationship between clinical demeanour score and rectal temperature is not linear. Once the pigs become mildly ill, increases in either of these two indicators of disease severity are more evenly proportional to increases in the other. However, healthy pigs also often have rectal temperatures which are measured as above 'normal', largely due to the stress involved in catching and handling pigs and taking their temperatures. This apparent rise in temperature due to handling stress should not be construed as being due to an increase in lung pathology.

Figure 9:



In each study, infection caused a decrease in feed and water intake, but there was no correlation with clinical severity by group. Therefore, although changes in food and water intake may be indicative of a disease, there is no apparent relationship between clinical signs or severity of pathological change and water or feed intake when measured on a group basis. This is possibly a reflection of the dynamics of the group and the lessening of competition for food or water for less affected pigs in the group. It is likely that if individual water or feed intake data could be collected, a relationship between increasing clinical and pathological signs and decreasing intake could be demonstrated, however under commercial situations pigs are housed in groups and in this situation no clear trend was detected.

Table 21a: Spearman's correlation coefficients quantifying the strength of correlation between indicators of disease severity in two experimental infections of pigs with *A. pleuropneumoniae* serotypes 3 and 5a

Study	Correlation coefficient (p-value)	Clinical score	Rectal temperature	Respiratory score	% Lung consolidation	Lung/bodyweight ratio (%)
PUK9501	Rectal temperature	-0.13 (0.30)	-	-	-	-
(n=58) *	Respiratory score	**0.52 (<0.001)**	0.11 (0.41)	-	-	-
	% Lung consolidation	0.05 (0.72)	0.10 (0.45)	0.10 (0.44)	-	-
	Lung/bodyweight ratio (%)	-0.04 (0.76)	0.25 (0.06)	0.19 (0.15)	**0.71 (<0.001)**	-
PBE9501	Respiratory score	-	**0.36 (0.02)**	-	-	-
(n=40)	% Lung consolidation	-	0.19 (0.24)	0.22 (0.16)	-	-
	Lung/bodyweight ratio (%)	-	-0.02 (0.88)	-0.03 (0.85)	**0.44 (0.005)**	-
	Bacteriological reisolation	-	0.24 (0.14)	0.09 (0.57)	**0.32 (0.04)**	**0.31 (0.05)**

Table 21b: Spearman's correlation coefficients quantifying the strength of correlation between indicators of disease severity in an experimental infection of pigs with *A. pleuropneumoniae* serotype 9

Study	Correlation coefficient (p-value)	Clinical score	Rectal temperature	Respiratory score	% Lung consolidation	Lung/bodyweight ratio (%)
PNE9501	Rectal temperature	0.02 (0.87)	-	-	-	-
(n=50)	Respiratory score	0.26 (0.07)	-0.11 (0.44)	-	-	-
	% Lung consolidation	0.05 (0.72)	**0.34 (0.02)**	-0.06 (0.66)	-	-
	Lung/bodyweight ratio (%)	0.12 (0.42)	**0.33 (0.02)**	0.07 (0.62)	**0.79 (<0.001)**	-
	Cough score	0.11 (0.45)	0.27 (0.06)	0.06 (0.66)	0.27 (0.06)	0.22 (0.12)
	Weight gain (%)	-0.13 0.37)	-0.16 (0.27)	-0.07 (0.64)	-0.14 (0.34)	-0.18 (0.21)
	Tracheo bronchial lymph nodes	**0.29 (0.04)**	-0.02 (0.88)	-0.09 (0.55)	0.06 (0.67)	0.08 (0.56)
	Thoracic lymph nodes	0.18 (0.22)	0.00 (1.00)	-0.21 (0.14)	0.13 (0.36)	0.24 (0.10)
	Sternal aortic lymph nodes	0.26 (0.07)	0.19 (0.19)	0.10 (0.48)	0.27 (0.05)	0.23 (0.10)
		Cough score	Weight gain (%)	Tracheo bronchial lymph nodes	Thoracic lymph nodes	Sternal aortic lymph nodes
	Weight gain (%)	-0.08 (0.60)	-	-	-	-
	Tracheo bronchial lymph nodes	0.24 (0.09)	**-0.33 (0.02)**	-	-	-
	Thoracic lymph nodes	0.16 (0.28)	**-0.32 (0.02)**	**0.53 (<0.001)**	-	-
	Sternal aortic lymph nodes	0.23 (0.11)	-0.26 (0.06)	0.25 (0.08)	0.02 (0.89)	-

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Appendix

(supporting data to Chapter 3)

CONTENTS

LABORATORY STANDARD OPERATING PROCEDURE : GROWTH OF *MYCOPLASMA GALLISEPTICUM* R-PIO

Preparation of the inoculum: At least five days before challenge of chickens, one tube from the stock culture of *Mycoplasma gallisepticum* R-PIO kept at -70°C in 5 ml aliquots of mycoplasma FM4 broth containing 10% glycerol (Merck 4094) are thawed and inoculated in 4.5 ml of FM4 medium contained in a 15ml glass tube. The culture is incubated aerobically at 37°C for 48 to 72 hours until the colour of the medium changes from pink to yellow. Then two 250 ml glass flasks containing 50ml of FM4 medium are inoculated with 2 ml of the culture and incubated aerobically at 37°C for 24 hours until the colour of the medium becomes yellow.

Storage of the inoculum: the inoculum is stored at 5±3°C until inoculation.

Titration of the culture: before and after inoculation of the chickens, tenfold dilutions of the suspension are made in FM4 medium and incubated aerobically in 5ml plastic tubes at 37°C to determine the concentration of the suspension (Rodwell and Whitcomb, Methods in Mycoplasmology 1:185-196,1983).

Control medium: A control FM4 medium is prepared and consists of tubes and flasks containing sterile medium incubated under the same conditions. This sterile medium is used to inoculate non-infected control birds.

LABORATORY STANDARD OPERATING PROCEDURE : RECOVERY OF *MYCOPLASMA GALLISEPTICUM* R-PIO

Approximately one centimeter of the distal third of the trachea is sampled from every dead or euthanased chicken and placed in a plastic sterile tube containing 1 ml of transport medium. Each tube is identified with the chicken number. Tubes are maintained at 5±3 °C until the day of culture (not later than 72 hours later).

In the laboratory, all work is done under a laminar flow hood.

From each sample, an aliquot of 10µl is used to inoculate an FM4 agar plate and an aliquot of 0.250ml is used to inoculate 0.75ml of FM4 broth contained in one 5ml plastic tube (TO tube). Two tenfold dilutions are made from TO into FM4 medium. The three tubes (labelled TO, T-1 and T-2) and the agar plate are incubated aerobically at 37°C. Ten ml of broth are sub-cultured onto mycoplasma FM4 agar when there is a colour change. If no tube has changed colour after one week of incubation, 10µl of suspension from tube TO are seeded onto FM4 agar. All agar plates are incubated for 21 days and observed twice a week for the presence of mycoplasma colonies.

Identification of two isolates per replicate is performed according to their biochemical properties (metabolism of glucose and arginine, phosphatase activity and tetrazolium reduction).

TRANSPORT MEDIUM FOR MYCOPLASMA

Buffered peptone water (Merck 7228)	25.5g
Glycerol (Merck 4094)	12ml
Distilled water	1 litre

Adjust pH to 7.4-7.6

Sterilise by autoclaving 20 minutes at 121°C.

Aseptically add:

Specillin G: 106 IU

Thallium acetate (Merck 12635) filter-sterilized: 0.5g

The transport medium may be stored up to one week at 5°C±3°C or up to one year at -20°C

LABORATORY STANDARD OPERATING PROCEDURE : SLIDE AGGLUTINATION TEST FOR *MYCOPLASMA GALLISEPTICUM* SEROLOGY

Sera from 20 one-day old chicks and from all birds necropsied on day 21 were tested for *Mycoplasma gallisepticum* agglutinating antibodies, according to the method described in:

Nougayrede P., Toquin D., Andral B. and Guittet M. Avian Pathology, 13,753-768.

Mycoplasma gallisepticum antigen were obtained from Intervet.

Sera are first tested undiluted. Positive sera are then retested after being heated for 30 minutes at 56°C and diluted 1:5 with phosphate-buffered saline (PBS) pH 7. Those that still react are considered positive. Sera that react only when undiluted are considered as as uncertain results.

INOCULATION OF BIRDS

On day 10 of life, birds are individually inoculated with a *Mycoplasma gallisepticum* culture prepared according to SOP "Growth of *Mycoplasma gallisepticum* R-PIO" . Each bird is inoculated with 0.2 ml intra-tracheally and with 0.05 ml into the right sinus.

Material requirements for intra-tracheal inoculation include a good source of light, IMI pipets equipped with thin tips and paper towels.

For intratracheal inoculation when the glottis is opened, the pipet is inserted into the trachea and the bird is inoculated.

Material for inoculation into the sinus include PIOO Pipetman with appropriate sterile tips. The bird is held on its left side, on a table 0.05ml of culture is inoculated into the right nostril. The birds are maintained in this position until the drop disappears.

Control (non-inoculated) birds are similarly inoculated with sterile FM4 broth.

STATUS OF SPECIFIC-PATHOGEN-FREE CHICKS

Specific-pathogen-free chicks (SPF) are obtained from SPF flocks

These flocks are repeatedly tested to ensure freedom from the pathogens listed below:

Avian adenovirus
Avian reovirus
Avian encephalomyelitis virus
Infectious Bronchitis virus
Gumboro (Infectious Bursitis) virus
Avian orthomyxovirus (type A)
Avian infectious laryngotrachyitis virus
Marek's Disease virus
Newcastle Disease virus (Avian paramyxovirus type 1)
Infectious anaemia virus
Mycoplasma gallisepticum
Mycoplasma synoviae
Salmonella pullorum

EXPERIMENTAL ANIMAL HOUSE

The Experimental Animal House was purpose-built for the housing of poultry in isolation for experimental work and for the containment of pathogens. Appropriate measures are taken to prevent access of rodents, wild birds, insects and unauthorised people. The building is supplied with a filtered air supply, performed by one single high efficiency particulate air filtration system (HEPA 99.9% efficacy) on the inlet supply and three HEPA filters on the extraction system. The building includes 16 animal rooms, 25m^2 each, light-, temperature- and ventilation-controlled. Each room is equipped with an air-lock chamber and with a shower-through entry system. Birds are housed in filtered air under positive or negative pressure according to their status (SPF birds or infected ones). Each room is also equipped with two HEPA filters on the inlet supply and three HEPA filters on the extraction system.

Before entering a non-infected room, personnel shower in the air-lock chamber and change clothing (including, hair protective clothing) and shoes. Before entering an infected room, clothes are changed in the lock chamber and, after visiting infected birds, it is necessary to shower in the air-lock chamber. Contaminated clothes remain in the contaminated room.

Items taken into the room are sterilised or put in plastic bag before entering the room.

Food is sterilized by granulation, and is wrapped up in plastic bag and paper bag. The bag is carried to the experimental animal house where it is fumigated after elimination of the paper bag.
Drinking water is tap water, heated to 80°C and then cooled.

After completion of a trial, rooms are cleaned and sanitized. All feed and equipment is fumigated into the animal room. Litter, feed and carcases are incinerated within the building and liquid waste is treated with sodium hydroxide.
Chickens are kept in two batteries of cases on three decks and two rows. Ten birds are kept in each cage.